University of Alberta

INTRAVESICAL THERAPY OF SUPERFICIAL BLADDER CANCER: IMMUNOTHERAPY AND VIRAL ONCOLYSIS

By

Erich Gregory HANEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE

In

Experimental Surgery

Department of Surgery

Edmonton, Alberta

Fall 2002

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-81417-3

Canada

UNIVERSITY OF ALBERTA

Library Release Form

Name of Author: Erich Gregory Hanel

Title of Thesis: Intravesical Therapy of Superficial Bladder Cancer: Immunotherapy and Viral Oncolysis

Degree: Masters of Science

Year this degree granted: 2002

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

(Signed) Erich Hanel

301-10548 78 Avenue

Edmonton, Alberta

Canada, T6E 1P4

Date: August 23/02

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify they have read and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "*Intravesical Therapy Of Superficial Bladder Cancer: Immunotherapy and Viral Oncolysis*" submitted by Erich Gregory Hanel in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

Ronald B. Moore, Supervisor

Patrick Lee

Louis H. Honoré

Greg Korbutt

Date: Sept 28, 2002

ABSTRACT

Superficial bladder cancer represents the majority of diagnosed bladder cancer. The best treatment is resection of the tumor followed by intravesical therapy. Bacillus Calmette Guerin (BCG) is the current standard. Approximately 20% of BCG therapies fail and serious complications can develop. Intratumor injections of oncolytic viruses have proven effective. Transformed cells with dysfunctional Ras kinase are selectively lysed by mammalian reovirus (REO). Bladder cancer cells were tested for susceptibility to reovirus and assayed for Ras activity. Using a rat bladder cancer model, a randomized dose escalation of intravesical REO was used to measure survival and side effects compared with BCG. Bladder cancers do express Ras proteins and are lysed by REO. Survival was increased in REO treatments relative to other therapies. Histology showed limited side effects in REO treated animals compared with BCG treated animals. These results support further clinical studies into REO as a bladder cancer therapy.

DEDICATION

To my family,

My supervisor,

My many mentors,

Many unrecognized support staff,

My friends who are always supportive,

The University of Alberta

Department of Surgery,

Division of Urology,

My amazing wife Meredith,

And Max (the dog).

ACKNOWLEDGMENT

It took the collaborative effort of a focused research team under dedicated guidance to allow me to complete this research project. First and foremost I need to thank my support network including my family (Florence and Werner Hanel and my brother Norman), my wife Meredith and all my friends. My supervisor, Dr. Ronald Moore proved to be a great support throughout my project. Not only in teaching me proper scientific method but also motivating me to pursue both clinical and research medicine. He is an example of what I hope to be, excelling in both science and medicine. My supervisory committee including Dr. Ray Rajotte has been excellent in supporting and shaping my research. Dr. Greg Korbutt's attendance and input at my defense on Dr. Ray Rajotte's behalf was appreciated. My mentors always pointed out the good and bad aspects of all my ideas. My thesis not only included basic translational research, but also clinical material. Courses at the University of Alberta gave me the opportunity to be taught by great professors. These include Dr. Garth Warnock and Dr. Aziz Ghahary. Special thanks go out to Dr. Ruhangiz Taghi-Kilani, Dr. Yahya Tamimi and Dr. Zhengwen Xiao for all their help and guidance.

Financial support for my project came from a variety of sources. These include Aventis Pharmaceuticals Inc and Oncolytics Biotech Inc. Both biotech industries provided me with more than funding, in many times they offered guidance and unbiased support. The Alberta Heritage Foundation for Medical Research also funded me as a summer student during the summer of my defense.

TABLE OF CONTENTS

INTRODUCTION 1

BACKGROUND 7
- Etiology of Superficial Bladder Cancer 7
- Superficial Bladder Cancer Classification 8
- Diagnosis of Bladder Cancer 13
- Management of Superficial Bladder Cancer 18
- Bacillus Calmette-Guerin (BCG) 24
- Oncolytic Viruses 33

PROPOSED RESEARCH PROJECT 42

MATERIALS AND METHODS 43
- Preparation and Culture of Rat TCC 43
- *In Vitro* Kinase Analysis 43
- Western Immunoblotting 44
- MTT Cytotoxicity Analysis 45
- Immunohistochemistry 45
- Multicellular Spheroid Analysis 46
- Animals 47
- Tumor Cell Implantation 51
- Urine Cytology 52
- Intravesical Therapy 53
- Mammalian reovirus serotype 3 56
- Histological and Statistical Analysis 56

RESULTS 58
- *In Vitro* Kinase Analysis 58
- *In Vitro* Reovirus Cytotoxicity 60
- Immunohistochemistry 65
- MCS Co-Culture TCC REO Assay 67
- Transplantable Orthotopic Bladder Tumor Model Implantation 69

Animal Studies 72
- BCG Intravesical Therapy (Dose Response Study) 72
- Reovirus Intravesical Therapy (Dose Response) 74

DISCUSSION 82

References 100

LIST OF TABLES

Table 1. Associated risk factors for bladder carcinogenesis..... 4

Table 2. The signs and symptoms of bladder cancer in an experimental animal model. The common signs in humans include hematuria and dysuria. The use of cytology has not been reported/investigated in the animal model. 14

Table 3. Cytological features of bladder cancer. 16

Table 4. A. BCG intravesical treatment groups. B. REO treatment groups. All treatments start at day 10 post implant. 54

Table 5. Relative risk (RR) calculated for all intravesical treatment groups relative to Sham saline controls. Comparative p values are included for each group compared with controls using a Chi squared test. All REO groups are statistically significant. Half frequency BCG instillations (1x6) were farthest from statistical significance. 77

Table 6. Summary of necropsy and histology of treated animals. For cytology, - means negative, / means not done or non-diagnostic, + means positive. Not all animals were included in the histological blinded analysis. HN means hydronephrosis. Tumor indicates presence of tumor, stone means a bladder stone was found. Lymphocyte indicates there are obvious signs of neutrophil infiltration. Red blood cells (RBC) were recorded..... 80

LIST OF FIGURES

Figure 1. Molecular transformation pathways of bladder cancers. Two main types of superficial bladder cancers occur, papillary and flat *in situ* disease. Adapted from Lee, R. and M.J. Droller, The natural history of bladder cancer. Implications for therapy. Urol Clin North Am, 2000. 27(1): p. 1-13, vii. 5

Figure 2. TNM (1997) Stage and Classification of Superficial Bladder Cancer. Superficial bladder cancer never penetrates more than the first layer of the bladder. Tumors that advance through the lamina propria into the muscularis are considered the more rarer advanced bladder cancer. 10

Figure 3. Kinase signaling and the interrelationship with REO oncolysis. Positive arrows indicate progression while blunted (square or round) indicate inhibition. Both receptor mediated cell entry and sub-viral entry are indicated. Apoptosis pathways occur after viral proteins are produced..... 38

Figure 4. Implantation regime for a transplantable, orthotopic superficial bladder cancer tumor model..... 49

Figure 5. A. Experimental setup for tumor cell transplantation and intravesical therapy. B. Gross features of a typical mid to late stage papillary bladder tumor in an untreated rat (~1 cm all dimensions)..... 50

Figure 6. Protein kinase activity determined using a functional assay. Units of kinase activity were calculated by using a standard PKC control set. Standard error isobars are included..... 59

Figure 7. AY-27 TCC survivorship tested using an MTT cytotoxicity assay. The cells were exposed to increasing REO concentrations for a 3 day incubation period. 62

Figure 8. A. TCC cell survivorship measure by an MTT cytotoxicity assay to varying levels of REO infection (MOI) using a 3 day exposure. Bladder carcinoma lines examined were AY-27 (rat) and MGHU3 (human). B. Complimentary western blot analysis of cell lines for raf-1 protein expression, a possible contributing factor to REO oncolysis. Loaded amounts of protein ranged from 15-70 □g. 63

Figure 9. Dose response for varying levels of REO using AY-27 TCC using an MTT absorbency assay (measure 570 nm and reference 680nm). Both virus exposed and negative controls (non-virus exposed) are compared. 64

Figure 10. Immunohistochemistry to detect REO particles in AY-27 TCC. Exposure times are indicated in each panel up to one week. An initial dose or REO $5x10^7$ PFU was used to parallel the high dose used in *in vivo* experiments. Staining was not optimal and quantification could not be performed. Light microscopy did reveal normal contacts at day 1, malignant cells would break up and dispurse as early as day 3, massive cell death by day 5 with evidence of apoptosis and complete cell destruction of monolayers by day 7 (as indicated in the figure). 66

Figure 11. A. IHC detection of TCC (MGHU3) and fibroblast cells (CRL-1120) in a MCS co-culture system. B. Selective viral oncolysis indicated by vital dye staining of MCS co-culture. Peripheral MGHU3 are selectively destroyed at increasing time points (indicated in days) evident by red staining. Central CRL-1120 are alive at each time point evident by green staining. 68

Figure 12. Typical urine cytology results for the AY-27 TCC animal model. Urine collection times are indicated for each implanted animal. Classic signs of positive cytology are evident by day 10 (increased cell number, N/C ratios, malignant cell clustering). 71

Figure 13. Kaplan-Meier (K-M) survival curves for BCG treated animals. Half-frequency BCG treatments $1x10^6$ CFU, low dose $1x10^5$ CFU, medium dose $1x10^6$CFU, high dose $1x10^7$ CFU, and IL-2 combination therapy $1x10^6$ CFU + $1x10^5$ U IL-2. 73

Figure 14. Kaplan-Meier (K-M) survival curves for REO treated animals. Low dose REO $1x10^5$ PFU, medium dose $1x10^6$ PFU, high dose $1x10^7$ PFU. 75

Figure 15. Kaplan-Meier (K-M) survival curves for optimal treatment regimes. Medium dose BCG and REO are included and compared with Sham controls. 76

Figure 16. Histology composite of experimental animals. Treatments are indicated in each panel. Pertinent features include the nature of the untreated tumor, inflammation and tumor in BCG treatments and normal bladder architecture and lack of inflammation in REO treated animals. The Saline panel shows a high grade carcinoma. The 1/2 BCG panel shows a high grade carcinoma. BCG shows a carcinoma *in situ*. The IL-2 panel shows an invasive carcinoma. The REO panel indicates normal bladder architecture with no signs of inflammation. All images are at 20x magnification. 81

ABBREVIATIONS

BCG	Bacillus Calmette Guerin
REO	Orthoreoviridae
IL-2	Interleukin-2
LAK	Lympokine Activated Killer
Ras	Rat Associated Sarcoma (Protein Kinase)
Raf	Rat Serine Threonine Kinase
F344	Fisher F344 Laboratory Research Rat
IFN	Interferon
PDT	Photodynamic Therapy
MAPK	Mitogen Activated Protein Kinase
ERK1/2	Extracellular dependent Kinase
PKR	RNA dependent Protein Kinase
PKC	Protein Kinase C
AY-27	Rat TCC Cell Line
TCC	Transitional Cell Carcinoma
T1	Superficial Bladder Cancer Clinical Stage 1
Ta	Papillary Cancer Clinical Stage A
Tis	Carcinoma *in situ*
MTT	Methylthiazolyldiphenyl-tetrazolium-bromide
MGH-U3	Human Bladder TCC Cell Line
N/C	Nuclear-to-Cytoplasmic Ratio
MRI	Magnetic Resonance Imaging
TUR	Transurethral Resection
TURBT	Transurethral Resection of Bladder Tumor
TMN	Tumor-metastasis-node
MOI	Multiplicity of Infection
PFU	Plaque Forming Units
CFU	Colony Forming Units
mAb	Monoclonal Antibody
pAb	Polyclonal Antibody
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
IHC	Immunohistochemistry
MCS	Multicellular Spheroid
PSA	Prostate Specific Antigen
CCAC	Canadian Council on Animal Care
GLP	Good Laboratory Practices

INTRODUCTION

In this thesis I review our current understanding on the treatment of bladder cancer and present my research on developing a novel intravesical treatment with oncolytic reovirus (REO). Rationale and preclinical *in vitro* and *in vivo* data will be presented to support further clinical application of this treatment strategy. This work is a translational project looking at REO efficacy in an animal bladder tumor model. Both side effects and efficacy were examined. The ultimate goal is for this research to serve as the basis for phase I/II clinical trials using REO as a bladder cancer therapy and secondarily to provide some basic research information on REO as an oncolytic agent.

Superficial bladder cancer or transitional cell carcinoma (TCC) represents the majority (up to 90%) of diagnosed bladder cancer in the United States and Canada [1, 2]. At times more than just the bladder is affected, with entire areas of transitional cells (urethra, renal pelvis, etc) being involved [3, 4]. In contrast, in Egypt more than 90% of bladder cancers are squamous cell carcinoma due to endemic parasitic infestation (*Schistosoma haematobium*) [5]. Annually in Canada and the USA combined, there are approximately 55,000 new bladder cancers diagnosed when combined [6]. The incidence of bladder cancer is increasing and it is now the fourth most common malignancy in males, and the eight most common in women [6]. This trend of higher incidence of bladder tumors in men is a worldwide phenomenon and is not entirely explained by environmental factors. For example even though men more often work in an environment that may expose them to bladder carcinogens, women more often develop persistent or chronic cystitis that can also destabilize the genomic DNA. Bladder cancer is the second most frequently diagnosed urological malignancy secondary only to prostate cancer. The

lifetime risk of developing bladder cancer is 1.85% for men and 0.8% for women [7]. Of the 4,550 cases of bladder cancer in Canada (1998) approximately 1400 of these were fatal [6]. Although bladder cancer incidence is increasing, mortality is not rising proportionally. Incidence of bladder cancer in the United States increased 36% in the period of 1950 to 1990 [6]. Mortality rates from bladder cancer have decreased between 1980 and 1995. This may be due to a variety of factors, including early diagnosis, intravesical treatment strategies and aggressive treatment. Even with bladder cancer related deaths decreasing, it still remains a significant source of morbidity for thousands of people each year, with the final treatment option being cystectomy (removal of the bladder and associated pelvic organs) [1, 8, 9].

There are significant differences in bladder cancer incidence among races and social economical demographics. As well superficial disease appears to be distinct from invasive disease. This has lead epidemiologists to believe there are multi-factor genetic processes and/or genome damage involved [7, 10]. Like all tumors, bladder cancers are genetic in origin, i.e. a disease of genomic expression or alteration of the deoxyribonucleic acid (DNA) and are associated with a variety of risk factors (table 1). There have been rare cases of familial bladder tumors but more evidence is provided for environmental causes [11, 12]. Combination of genetic predisposition with environmental risk is a serious predictor for bladder cancer development [3, 13]. Transformation is believed to be a multiple step process involving repeated damage to the genome of transitional cells of lining of the bladder Carcinogenic compounds are a leading cause of bladder cancer, initiating genetic damage at the cellular level [3]. Most of the suspect carcinogens can be found in tobacco smoke. Other compounds that are know transitional

cell promoters include aniline dyes and aromatic amines [14]. Parasites such as bacteria and viruses can lead to cystolithiasis, which can also transform the bladder lining. The molecular changes caused by carcinogens can include a variety of cell growth and differentiation pathways (figure 1).

Table 1. Associated risk factors for bladder carcinogenesis.

Occupational Risks
Dye workers
Textile workers
Tire and rubber workers
Painters
Truck drivers
Petroleum workers
Hairdressers
Aluminum workers
Chemical Carcinogens
2-Napthylamine
Benzidine
4-Aminobiphenyl
Nitrosamines
Cigarette Smoking
Miscellaneous Causes
Phenacetin use
Cyclophosphaminde treatment
Pelvic irradiation
Genitourinary tuberculosis
Chronic cystitis

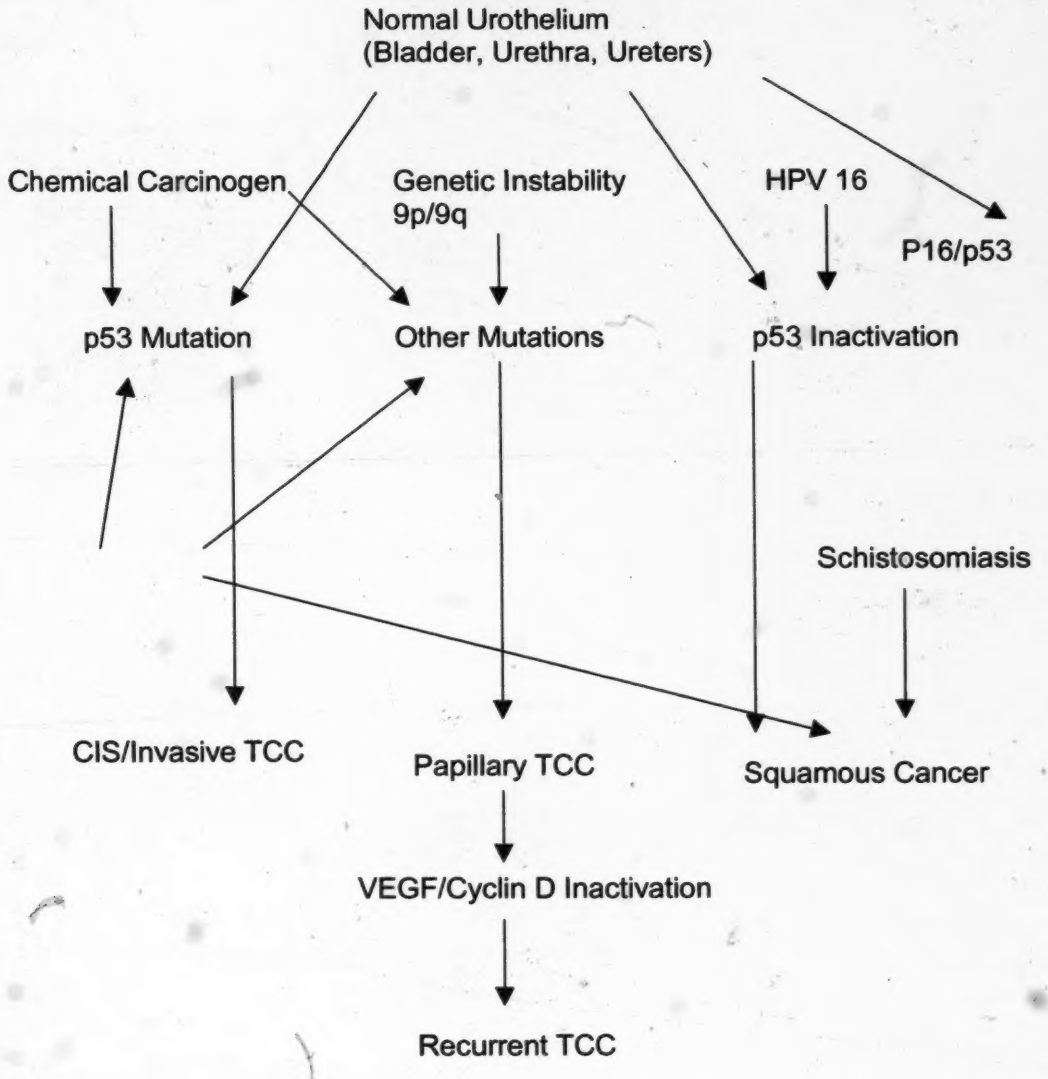


Figure 1. Molecular transformation pathways of bladder cancers. Two main types of superficial bladder cancers occur, papillary and flat *in situ* disease. Adapted from Lee, R. and M.J. Droller, The natural history of bladder cancer. Implications for therapy. Urol Clin North Am, 2000. 27(1): p. 1-13, vii.

Suspected mutations involved in bladder cancer transformation include cell cycle regulators (ERBB-2, MDM2, cyclin D1). On a larger scale, chronic irritation of the bladder (cystitis) can lead to bladder cancer, including squamous cell carcinoma (often from schistosomiasis parasites) [15]. Smoking however is the leading factor contributing to bladder cancer incidence [16]. Causative agents in cigarettes are the focus of current uro-oncology research. A chronic smoker faces up to 3 times the risk of developing superficial bladder cancer [16]. Cessation of smoking can reduce overall risk. Known bladder cancer carcinogens included, tobacco, beta-naphthalene, cyclic hydrocarbons, and other organic compounds [5]. Besides promoting cessation of smoking and reduction in carcinogen exposure, better strategies for early diagnosis and treatment of bladder cancer are needed. Most carcinogens are processed in the liver and then metabolized prior to excreted through the urinary system. This provides access of the carcinogen to the urothelium, often in close contact for long periods of time often many years.

The fact bladder cancer incidence increases with age can be partially explained by evidence that carcinogens accumulate and cause additive damage to transitional cell DNA ultimately leading to cell transformation and carcinogenesis. Beyond the age of 60, the overall risk of developing bladder cancer dramatically increases. Younger patients can develop bladder cancer but most often in these cases the tumor will be low-grade papillary and has an excellent prognosis.

BACKGROUND

Etiology of Superficial Bladder Cancer

Multiple theories for the development and progression of bladder cancer have been debated. These models for the etiology of bladder cancer have been hypothesized based upon clinical research and basic science into the molecular biology of bladder tumors. Diverging pathways of bladder cancer development have been proposed. One being more papillary and the other being flat (sessile), more aggressive and invasive [3, 17]. By far the most common type of bladder cancer (75%) is the superficial papillary lesion that seldom develops into an invasive tumor. The second class of tumor is a more solid mass or carcinoma *in situ* that has a great preponderance to develop into an invasive or metastatic tumor. These tumors have a high propensity to recur causing patients to submit to lifelong follow up for recurrent tumors. There are clinical predictors for recurrence, supporting different routes of disease development. These predictors include superficial invasion, high grade (grade 3), Tis, masses larger than 3 cm or previous recurrences [8]. A recurrent tumor may be due to inherent genetic damage in basal cells. As tumors are removed or treated, subsequent carcinogenic promotion, transformation or initiation of previously healthy cells can lead to recurrence. Molecular classification of tumors may help define which class of malignancy a patient has and help to predict tumor recurrence more accurately. Currently clinical based classifications have been developed as either being low, medium or high risk for more aggressive or recurrent disease [7, 18].

The natural history of bladder cancer has promoted investigations into gene mutations. This has included examining overexpressed oncogenes, and underexpressed tumor suppressor genes. Epigenetic factors such as parental DNA methylation may also

play a role in bladder cancer development and may shed light on differences seen universally in bladder cancer incidences in men and women [19]. Two models of carcinogenesis have emerged, one being the "field defect" model and the other is based on a clonal system [7, 17, 19-21]. The field defect model states that the urothelium may entirely be affected as a whole by mutating carcinogens and depending on a cells initial genotype, these mutational insults may cause transformation to varying degrees. Ultimately this would explain the multifocal nature of bladder tumors, and also the fact many will recur [22]. The clonal model in contrast states that single mutated cell populations thrive as tumors in the urogenital system [23, 24]. Through X chromosome inactivation studies (Barr bodies), patches of cell populations in the bladder (120 mm^2) can be identified. Using this sizing of cell patch colonies, it follows that the urothelium is only made up of an initial 300 cell clones [22, 25]. Mutations in these initial populating cells can lead to carcinogenesis. This would not explain the development of upper urinary tract cancers, which can be seen with initial bladder TCC. Normally urine and hence urothelium migration does not follow a retrograde pattern (from bladder to ureters and the kidney). However there is evidence for seeding of the bladder from upper urinary tumors.

Superficial Bladder Cancer Classification

Superficial bladder cancer is defined according to the 1997 System International TMN (tumor-metastasis-node) classification as Ta, Tis, and T1 (figure 2). The TMN classification of superficial bladder cancer limits the lesion such that there is no involvement of the muscularis propria. Tis, also known as carcinoma in situ (CIS), is a superficial lesion found at the external surface of the mucosa lining. CIS can involve the

entire mucosal layer. Ta bladder cancer projects into the bladder (papillary) and involves the mucosal layer. T1 bladder cancer also involves projections into the bladder, along with basal invasion of the lamina propria to but not including the muscularis propria. Any tumors in the bladder more severe than T1 or Ta is considered to be invasive and requires a different treatment approach as these cases may have malignant tissue beyond the bladder (nodes or distant site metastases). Localized treatment of only the bladder in these cases is not the best option, as tumors beyond the bladder can be fatal [8, 26]. Therapy targeting superficial areas of the bladder may not reach already invasive cells that have penetrated into the muscularis propria. Treatment for these cases will usually involve surgery, chemotherapy, radiotherapy or a multidisciplinary approach dependent on the overall health of the patient [27].

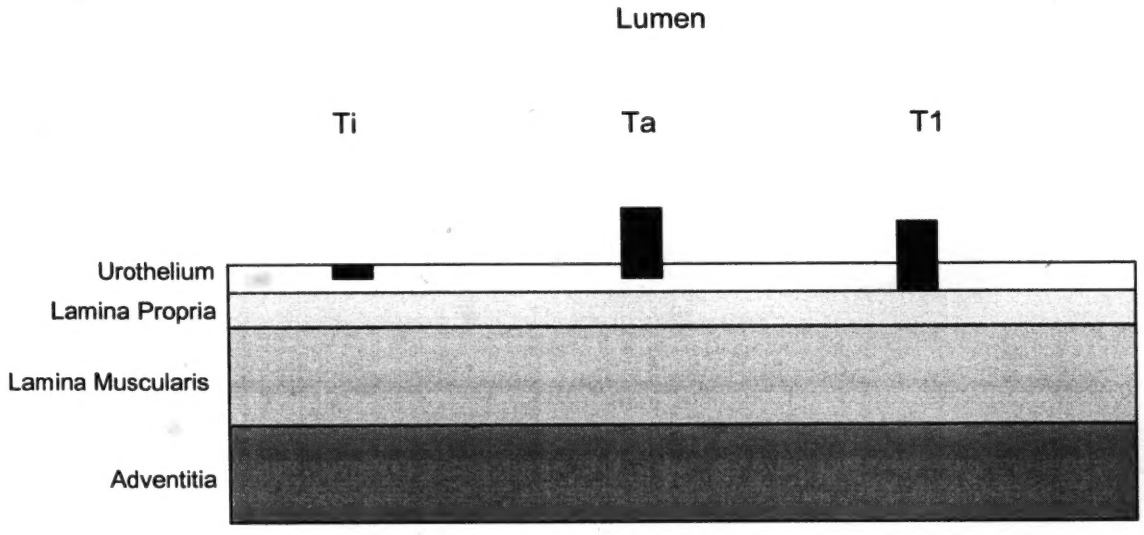


Figure 2.TNM (1997) Stage and Classification of Superficial Bladder Cancer. Superficial bladder cancer never penetrates more than the first layer of the bladder. Tumors that advance through the lamina propria into the muscularis are considered the more rarer advanced bladder cancer.

Three risk groups of superficial bladder cancer have been defined. The risk groups were initially classified into low, medium and high [7, 18]. Vicente-Rodriguez *et al* defined the groups as low being grade 1 stage Ta disease and a single grade 1 stage T1 tumor. Intermediate is defined as multiple grade 1 stage T1 tumors, grade 2 stage Ta disease and a single grade 2 stage T1 tumor. Finally high risk superficial bladder cancer is defined as multiple grade 2 stage T1 tumors, grade 3 stages Ta and T1 disease, and any stage disease associated with carcinoma in situ. Relative risks, disease progression and survival have been statistically established for these classifications. Recurrence risks for each group are (from low to high), 37%, 45% and 54% [7]. Progression rates for low risk superficial bladder cancer are 0%, medium is 1.8%, and high is 15% (respectively). Mortality for low risk is 0%, less than 1% for intermediate and 9.5% in the high risk group. It is useful to consider these statistics when classifying bladder cancer in order to provide an adequate treatment strategy.

Predicting tumor recurrence or aggressiveness (behavior) is clearly dependent on grading and staging a newly diagnosed bladder cancer. Tumor characteristics that increase the chance for recurrence include high-grade tumors, multiple foci tumors, carcinoma in situ, and predisposing genetic damage [17, 23, 28, 29]. Any one of these characteristics may warrant the use of adjuvant therapy beyond surgery (local resection) in the management of the disease. If a patient has already had a prior history of bladder cancer then by virtue recurrent cancers will be at high risk for further recurrence. The genetic damage commonly associated with bladder cancer involves lesions to chromosomes 7, 9 and 17 [19, 23, 30]. Mutations in these chromosomes are known as

primary alterations as they are frequently found in many different types of cancers, often directly implicated in carcinogenesis. These genes are involved in cellular differentiation, cell division and/or cell death (apoptosis). Chromosome 17 (p53) alternations are found in more aggressive, higher grade CIS lesions [23].

Unstable chromosomes can experience later replicative genetic damage (errors) that could lead to sporadic tumors. Genome ploidy status can also predict tumor behavior. Diploid tumors tend to be less progressive, while aneuploidy is associated with a greater risk [31]. Treatment must keep in mind the etiology of the disease, the underlying damage to the genome, and that further mutagenic insult could result in recurrence or progression of the of tumor. The 5-year recurrence rate of transitional cell carcinoma (TCC) is as high as 80% [32-34]. Frequent surveillance of individuals after any treatment for TCC is crucial, as recurrence is a major concern.

Diagnosis of Bladder Cancer

Identifying bladder cancer can be done routinely using a variety of diagnostic tests. Early detection is important, but many bladder tumors grow insidiously and therefore are detected late when signs and symptoms develop. Signs of bladder cancer in animals are specific but follow similar patterns seen in humans (table 2). Hematuria is a hallmark sign of bladder cancer and often is the initial presenting symptom. Gross total painless hematuria (GTPH) is the leading initial symptom of bladder cancer. Approximately 15% of all patients presenting with GTPH will have bladder cancer [35, 36]. GTPH presents with blood evident in the urine throughout micturition.

Table 2. The signs and symptoms of bladder cancer in an experimental animal model. The common signs in humans include hematuria and dysuria. The use of cytology has not been reported/investigated in the animal model.

Signs and Symptoms of Bladder Cancer	
Experimental Animal	**Human**
EARLY	**EARLY**
Gross/Micro hematuria Positive cytology	Gross/Micro hematuria Positive cytology
LATE	**LATE**
Limited movement Limited drinking Limited eating Piloerection Hunching of hind limbs Dragging of hind limbs Porphyryn ocular staining Decreased body mass "Scruffy" fur with poor grooming Pale skin (limbs and nose) Palpable mass	Dysuria Positive cystoscopy Weight loss Irritative voiding Urgency Frequency Obstructive voiding Renal failure Anemia Palpable mass

GTPH is a sign that the blood in the urine originated from the bladder or upper tracts. Pain during micturition is a hallmark of inflammation (infection or calculus), that may or may not be associated with bladder cancer. Early or initial bleeding during micturition is a sign of urethral, including prostate involvement, and terminal bleeding during micturition indicates involvement of the bladder dome [36]. Patients presenting with GTPH must be further examined for signs of bladder cancer. Testing for hematuria is a simple analysis with low cost per test but large expense per year, reaching 150 million dollars in the United States [37]. Urinalysis and cytology accompany hematuria testing in suspect bladder cancer cases. Cancerous cells are sloughed from the bladder urothelium and exhibit characteristic features (table 3). Many new molecular diagnostic research techniques are also being investigated for possible clinical use. These however may be limited in application due to cost.

The current methods used for the clinical diagnosis of bladder cancer are patient signs and symptoms (GTPH). This sign is followed by a urine cytology analysis, which can include additional experimental molecular marker tests. Finally when all prior tests are positive cystoscopy is used to directly observe the bladder and remove a biopsy for pathological analysis.

Table 3. Cytological features of bladder cancer.

Microscopic Features
Increased cell number
Increased N/C ratio
Nuclear hyperchromasia
Nuclear pleomorphism
Coarse chromatin clumping
Irregular and thickened nuclear membrane
Mitotic figures
Occasional nucleolus
Necrotic background

Several genetic markers for tumorigenic bladder cells have been well documented and investigated. These markers include MIB-1, p53, bcl-2, EGFR and c-erb. Some of these markers are being used as diagnostic techniques in clinical trials [19, 38]. For example, p53 can be detected in sloughed cells found in voided urine samples. Currently BTA, NMP22 and FDP are approved for clinical use [19, 38]. Many other markers and test kits are being evaluated in clinical research.

Besides offering information on diagnosing bladder cancer, molecular markers for bladder tumors, also provide information on the genotype of the cancer. Molecular markers of malignancy can help direct or modify therapy and treatment options. Usefulness of these markers is a topic of current debate within clinical research. The use of molecular markers in the clinic will hopefully reduce the amount of cystoscope surveillance that is now required.

More research in this field is required, as single molecular markers are not as useful as multiple markers in predicting the behavior of superficial bladder tumors or response to therapy. Currently only conventional histopathological methods are used to evaluate bladder cancer. Though very accurate at diagnosing bladder cancer, these methods have limited value in predicting course and prognosis. Even a correct classification of grade and tumor stage is not a reliable predictor of tumor behavior in all cases. The new treatment modalities should be coupled to new diagnostic techniques that will in concert allow for better management of bladder cancer.

Molecular diagnostics has the advantage of giving more information regarding tumor behavior than basic histopathology. For example, a critical feature of a tumor's ability to survive in the body is how it can manipulate the extracellular environment. Tumor

invasion and progression can be enhanced by imbalances in the matrix metalloproteinase and their inhibitors [39]. This very imbalance can be used to predict the chances of tumor recurrence, which is a common feature of superficial bladder cancer.

The more molecular markers that are characterized for bladder cancer the greater the chance this science will contribute to the overall field of clinical oncology. The use of molecular diagnostics can reduce the amount of potentially traumatic tests performed on bladder cancer patients. Cystoscopy is an invasive procedure and in more complicated cases involving co-morbidity in the prostate can be potentially harmful [40]. Therefore the less it is performed the less chance of causing untoward trauma to the urethra. Screening for bladder cancer is not recommended and generally only high-risk individuals are tested based on signs and symptoms and potential for bladder cancer. The gold standard of detection to date, remains cystoscopy, which is usually conducted if the patient presents with signs and symptoms of possible bladder cancer (hematuria and/or positive urine cytology) [18].

Management of Superficial Bladder Cancer

The general consensus regarding the management of superficial bladder cancer involves resection and the use of adjuvant immune modulation using intravesical Bacillus Calmette-Guerin bacteria (BCG) for prophylaxis and treatment of high risk of superficial tumors [8, 41]. This method of treatment has been compared to conventional cancer treatments such as intravesical chemotherapy. Though effective as both secondary and primary cancer therapies, these intravesical therapies do have the potential for serious side effects. BCG, although an attenuated mycobacterium, is a living organism capable of systemic infiltration. Antimitotic chemotherapy are themselves potentially mutagenic and

can further destabilize the urothelium. This, however, has not yet been born out in large studies of adjuvant intravesical chemotherapy [42].

Immunotherapy using BCG instillation is currently considered the gold standard of prophylactic superficial bladder cancer management and treatment for bladder carcinoma in situ (CIS) [43]. Intravesical BCG is also used to treat recurrent TCC. This method of immune modulation is based on the inflammatory response to a bacterial exposure. Intravesical administration of such a pathogen for tumor therapy has the advantage of being selective, but it is indirect and not necessarily specific. The mechanism of tumor destruction is dependent on the patient's immune response. The effective component appears to be the cell wall, which has been used in clinical trials (Regressin®) [44]. As well modulation of immune response with cytokines has been investigated [45]. Naturally occurring viruses capable of oncolysis may prove to be an optional treatment for superficial bladder cancer. An oncolytic virus administered into the bladder has the potential advantage of being selective as well as specific by lysing only tumor cells. Prior to this research project, there were no reports on the use of oncolytic viruses for instillation therapy for superficial bladder cancer. Further study is required into the mechanism of action of these intravesical biological treatments (viral or bacterial). In particular, research should examine cytokine modification of BCG therapy and compare this to novel intravesical oncolytic viral therapy.

The goal of any cancer therapy is to eliminate or reduce the number of tumor cells in a tumor. This should be accomplished with minimal risk to the patient, sparing healthy tissue to reduce morbidity. With modern medical fiscal restraints, it is critical to do this as economically as possible and ideally the therapy should be available to all patients.

Current management strategies for TCC currently include a combined modality management, and in most cases this includes surgical resection of the bladder tumor. This has been clinically proven to provide the highest level of bladder preservation with the lowest morbidity and mortality. Late stage bladder and metastatic cancer is treated with more aggressive (surgical intervention, radiation therapy and or chemotherapy, i.e. Cisplatin) treatments than those outlined here [27]. The following therapies include only superficial bladder cancer treatments.

Intravesical Chemotherapy

The bladder offers a unique opportunity for a more direct delivery of chemotherapeutic agents than the more common systemic route of drug administration. Drugs can be administered via a catheter inserted into the urethra. Effectively, intravesical administration of chemotherapy is a topical application of an agent to the tumor and surrounding bladder urothelium. The aim of intravesical chemotherapy is the same for cancer therapy in any organ system. The goal is eradication of the tumor with minimal discomfort or damage to surrounding healthy tissue

Intravesical therapy can involve administering treatment before the bladder tumors have a chance to grow to pathological sizes or to prevent recurrence after transurethral resection of the bladder tumor (TURBT). Intravesical chemotherapy offers marginal positive results with this goal in mind. The benefit of adjuvant intravesical therapy over TURBT alone is 14% during the first 3 years [8]. More than 50% had a decreased incidence of tumor recurrence with follow-up intravesical chemotherapy. To date, select patients treated with adjuvant therapy following TURBT have a better outcome than those treated by TURBT alone [8].

Chemotherapeutic drugs of choice for intravesical therapy include thiotepa, doxorubicin, mitomycin C and epirubicin [43]. These drugs do show a short-term benefit in terms of preventing tumor recurrence, but long term there has been no alteration in the progression of superficial bladder cancer to metastatic or advanced disease. Progression rates for control groups were actually lower than the experimental groups receiving the intravesical chemotherapy [43].

Intravesical chemotherapy alone versus surgical management of bladder cancer has a lower success rate [8]. Although the bladder may offer easy access of drug delivery via the urethra, it does not offer an accommodating environment for chemotherapy drugs. Urine production varies in times of health and disease and further varies between patients. This affects the volume and rate of bladder filling and emptying. Urine pH and electrolyte content can also affect chemotherapy used in the bladder [43].

Intravesical chemotherapy may not be a solution in itself for the management of superficial bladder cancer but in combination with other therapies it may improve patient outcome. In the treatment of CIS of the bladder, as many as 74% of patients may achieve complete response, although recurrence for these patients is still high [27, 43]. Consistent with other human cancers combined modality therapy appears to be the key to treating bladder cancer. The potential benefits include cytoreduction, prevention of seeding and elimination of the malignant phenotype.

Photodynamic Therapy

Photodynamic therapy (PDT) is a new experimental form of bladder cancer management that has been used clinically for the past decade only in select refractory cases and in clinical trials. The success of PDT depends on the pharmacology of non-toxic photosensitizers and laser physics. PDT involves treating the bladder tumor cells with a photosensitizer that becomes cytotoxic when exposed light provided at a specific wavelength from a laser source. Physically, PDT requires a laser fiber light source to be inserted into the bladder intravesically. Prior to this, the patient is treated with the non-toxic photosensitizer, using standard chemotherapy vectors and access routes (see below). The key to PDT is targeting the drug and the cytotoxicity to the tumor cells alone limiting damage to surrounding tissue and adjacent bladder cells. This would provide selective lysis of the cancer cells, sparing the surrounding healthy tissue. Currently PDT is recommended for papillary TCC, refractory Tis, and for prophylaxis of recurrent TCC for patients in whom intravesical chemo or immunotherapy has failed [46].

The photosensitizer can be administered systemically or locally instilled in the bladder. Liposomes or other delivery systems can further augment delivery of the drugs in the bladder. Side effects of systemically administered photosensitizers include cutaneous photosensitivity, and reduced bladder capacity (from non-specific cellular damage and scarring) [47]. Common photosensitizers are the porphyrins, napththalocyanines, purpurins, chlorins and bacteriochlorins; though Photofrin II is the only one approved for clinical trials [48]. Photofrin, besides being a photosensitizer has another chemical property. When excited with UV (400nm) or blue light (510nm)

Photofrin will fluoresce (600-700nm) which can offer the potential for visualizing flat (CIS) tumors with a cystoscope.

When injected intravenously (22-hour serum half-life) the majority (88%) of the Photofrin II is transported by lipoproteins and the remainder is transported by albumin [49]. Once the Photofrin is administered systemically it is selectively taken into the tumor by lipoprotein (LDL) receptors and possibly non-receptor mediated endocytosis cascade [49, 50]. LDL receptors are common to malignant cells as well as inflammatory cells. This accounts for the fact that 24 hours after injection of the dye into the body it will accumulate in the reticuloendothelial system as well as liver, kidney and spleen. Besides intravenous injection, photosensitizers can be instilled in the bladder to further increase the localization of the drug to the target tumor tissue. In animal models, bladder instillation had comparable results when compared to intravenous administration [50].

Clinically PDT has proven to be quite effective in the management of certain types of bladder cancer. In one study by Nseyo, a single PDT treatment achieved 84% success rate with patients suffering from recurrent superficial papillary TCC, 80% with refractory CIS and almost 90% in patients receiving PDT as prophylaxis bladder cancer management [46]. Whole bladder PDT treatment for TCC is recommended as a second line of therapy in BCG refractory TCC cases [1].

PDT is not limited only to the treatment of bladder cancer. Other areas where this cancer therapy may prove useful include; prostate, lung, uterus, head and neck, esophagus, and stomach [48]. In many of these areas, the application of PDT requires more research and refinement, but the potential exists for improving on current therapies.

Bacillus Calmette-Guerin (BCG)

Pearl, in 1929 reported an autopsy series demonstrating those patients with clinical tuberculosis had lower than average incidence of tumors [51]. It was proposed that an attenuated tuberculosis vaccine might have potential as a cancer therapy. Later in 1969, research showed that BCG held promise as a treatment for lymphoblastoid leukemia [52]. At the same time, radiotherapy and chemotherapy experiments promised much better efficacy than BCG and immunotherapy. Vaccination has been used in medicine for a variety of ailments but BCG is perhaps the first vaccination for cancer. BCG is an attenuated tuberculosis organism and current thought about its mechanism of action on tumor size reduction revolves around host immune system modulation. Animal studies using BCG clearly proved that there was a prolonged BCG inflammatory reaction in the urinary bladder. This inflammation has been associated with tumor reduction and cell clearance [53]. Dr. Morales successfully used BCG as an intravesical agent to treat superficial bladder cancer in 1976 [54]. Dr. Lamm, who performed the first randomized controlled clinical trial using BCG as an intravesical agent for superficial bladder cancer, followed up this study [55].

Since Dr. Morales' discovery, BCG has become the gold standard treatment for prophylaxis and management of CIS. As many as 80% of patients treated with BCG immunotherapy will have long-term success in the form of elimination of tumor cells or protection for tumor recurrences [1].

Before some of the current theories of BCG's mechanism of action where developed, it was well accepted that BCG therapy was dependent on several factors. These factors

include the amount of viable BCG used in treatment, the method of administration, tumor size and character, and finally the ability of the bacteria to adhere to the tumor [56, 57].

Intravesical instillation therapy begins anywhere up to 4 weeks after resection [1, 58]. There are several criteria candidate patients must fulfill. At the time of instillation they should have no bladder irritability and there should be no hematuria, both are common post-operative side effects. Patients ideally should have a normal urinalysis prior to initiation of therapy. Before instillation it is important to have minimal amounts of residual urine in the bladder. Residual urine in excess of 100 ml may cause increased exposure to BCG and this could aggravate side effects [59, 60]. At the initiation of BCG therapy the location, number of foci and sizes should be recorded in order to assess response. Males undergoing treatment should have their prostate specific antigen (PSA) levels measured before BCG therapy as instillation of BCG tends to increase PSA [61].

With prophylactic BCG therapy an initial induction of the immune system is necessary [62]. It is this immune system activity that is postulated to be the basis of BCG action. This is usually achieved after six weekly installations, though some studies have used weekly instillations for as short as two weeks [79]. Once a reaction in the epithelial lining of the bladder is achieved there is most likely no need for further instillations although there is a marked variation in patient response. The attending urologist can design a schedule of instillations based on the individual response. If a patient develops a severe reaction to the initial therapy, the following installations can be delayed without compromising efficacy of immune induction [9, 64, 65]. Delaying treatment may actually decrease side effects in these cases as a rapid inflammatory response followed by additional BCG may allow systemic spread. There has been variation in the amount of

BCG instilled and the use of maintenance instillation when the goal of therapy is prophylaxis [66]. There is a good chance of responding to a second BCG instillation once there has been a failure in response to initial therapy. As such BCG refractory TCC is defined as failure to respond to 2-6 weekly courses of BCG [26]

An active host immune system is essential for BCG to have any anti-tumor effect. BCG therapy involves several defined stages. Initially after intravesical instillation, BCG comes into direct contact with both healthy urothelium and transformed cells. Collagens, specifically fibronectin is required for BCG to make physical contact and adhere to healthy and tumor cells [56, 63].

Besides tumor destruction mediated by BCG induced local inflammation cytokines and other immune chemicals are thought to play a role. Nitric oxide (NO) is used by immune cells to destroy pathogens and aberrant cells. Research has shown that nitric oxide synthetase (NOS) activity and the production of NO have a cytotoxic effect [58, 67]. BCG induces both a calcium dependent and calcium independent production of NOS in the urothelium.

Candidates for BCG instillation therapy may be predisposed to launch an immune reaction to mycobacterium antigens [63]. Research by Huygen et al showed that patients about to undergo BCG therapy had a higher response to BCG immunogens [45, 63]. This suggests that bladder tumors may have similar antigenic epitopes as BCG. The implications of this are that BCG therapy represents a secondary exposure to TCC tumor antigens and this would be followed by a stronger secondary immune response leading to increased reactivity and increased tumor destruction.

Patients having BCG instillation therapy should be monitored for side effects and progress should be quantified. This can be achieved with urine cytology, which can be done any time after treatment. Cystoscopy can also be used to monitor patients but it is important not to traumatize the bladder or surrounding structures, which could potentiate side effects.

Many patients will have minor reactions to BCG therapy and as high as 5% will have more severe side effects [55, 68]. The more common side effects include increased frequency of urination with dysuria. These are also side effects experienced by some patients following cystoscopy. There are two main factors that contribute to the complications of BCG immunotherapy. First BCG is a living organism, though attenuated it has potential to grow and thrive in the human body. Second the goal of therapy is to bolster the patient's immune response and through local inflammation, destroy existing or potential tumor cells. Increased level of immune cell activity in the bladder, thought to be manifestations of the immune response elicited by BCG, can present as general flu-like signs and symptoms.

Early reactions to BCG therapy may occur after the third instillation [55]. Cystitis is usually the first to present. Along with cystitis there can be general malaise and mild fever. These are indications that the immune system has become locally active. A potentially serious complication seen in approximately one third of patients presenting with BCG induced cystitis is hematuria [55]. This can be dangerous as increased hemorrhage in the bladder and surrounding structures can provide BCG a route of systemic entry to the vascular system. There have even been several cases of miliary tuberculosis (TB) of the lung in patients undergoing BCG instillation therapy [69]. TB as

a side effect of BCG therapy is uncommon, occurring in 1-3% of patients [69]. The majority of BCG treated patients are free from adverse side effects (95%), though there is the risk for serious complications [55, 68, 70, 71].

Fever is one of the more common and potentially serious side effects of BCG therapy. When fever presents, usually after the third instillation, there can be two possible explanations. The fever may be a normal uncomplicated side effect of immunotherapy or it could be the early signs of systemic infection and severe BCGosis, which can lead to anaphylaxis [55]. Uncomplicated fever associated with BCG therapy will resolve in a couple of days with general antipyretic management. The danger with fever in patients undergoing BCG therapy is the fact that the uncomplicated fever or the early signs of systemic toxicity cannot be distinguished. Any patient presenting with fever during BCG therapy is hospitalized and treated with isoniazid and rifampicin [55, 70, 72]. Patients can resume BCG therapy after the fever resolves but the BCG is administered at a lower dose and is accompanied by prophylactic antitubercular antibiotics before instillation [73, 74].

Systemic infection of BCG can be fatal. There have been reported deaths due to BCG instillation therapy [55]. It should be noted that side effects of BCG vary among the strain of attenuated bacterium used. The Tokyo and RIVM strains have been known to be associated with greater risk of sepsis [75]. There is no statistical difference between strains of BCG with regards to treatment efficacy [43]. Because early management of fever can prevent sepsis it is important to note that fatalities of BCG can be avoided with proper management and monitoring during treatment. Large amounts of BCG are used for instillation therapy. The bladder is an open environment when contrasted to the intravenous environment. Because patients can void spontaneously, the amount and

duration of BCG can be quickly reduced in a patient with an unstable bladder. Therefore, the actual amount of BCG received by the superficial bladder cells will vary between patients.

Environmental factors can contribute to the internalization of BCG, this further randomizes the actual amount of BCG antigens exposed to the local immune system. The doses of BCG used for intravesical instillation would be fatal if administered intravenously. It is therefore critical to ensure that BCG does not have the option of entering the systemic circulation. This can be done by making sure instillation therapy is done at a time when hematuria is low and cystitis is not present. Most candidates for BCG instillation therapy will have prior bladder resections. There must be enough time between resection and instillation therapy so that the bladder has time to heal and there is not vascular access for the pathogen. Isoniazid and rifampicin can be used to treat mild BCGosis, but for more severe or advanced case cycloserine has been known to inhibit BCG growth very rapidly (within 24 hours) [9, 64, 76]. Another report on inhibiting BCG growth states that isoniazid and rifampicin combined with prednisolone is the best option, which is currently the treatment regime of choice [59].

The actual administration of BCG during instillation therapy must be done soon after the lyophilized BCG is reconstituted to keep the number of viable mycobacterium high. Initial BCG CFU are calculated prior to packaging, and expiration dates should be adhered to. The amount of viable BCG in an instillation is critical to success of therapy [64, 77, 78]. Too much BCG (10^9 cells or more) can not only lead to increased chances of systemic side effects but also can overwhelm the immune system (anergy), which can

enhance tumor growth. Alternatively, a lack of viable BCG (10^6 cells or less) may not elicit the desired inflammatory response in the bladder [79].

Trauma during instillation to the bladder or any of the genitourinary structures should be avoided before instillation of BCG, as this is associated with higher rates and increased severity of side effects. Trauma allows a potential route for BCG to enter the systemic circulation, which could lead to widespread sepsis including complete BCG infection, known as BCGosis [74, 80]. Once instilled in the bladder the solution should ideally be retained for a minimum of 2 hours. The patient should be advised to use a single toilet in their home for the 24 hours following BCG instillation and should refrain from sexual activity for 48 hours. To date, there have been no reported cases of patients treated with BCG inoculating members of their family or the general public.

BCG is a useful treatment after transurethral resection of the bladder for prophylaxis against tumor recurrence. Patients with stage T1 tumors of any grade, high grade Ta lesions or patients with multi-focal or recurrent tumors or genetic predisposition have a high chance of recurrence [33, 81, 82]. Post-surgical instillation therapy in these patients is often beneficial. The majority of patients with low grade Ta lesions do not benefit from instillation therapy. These patients represent 20% of all diagnosed bladder cancers. Grade 1 Ta lesions are characterized with a 2% progression rate and usually do not progress to invasive levels [33, 83].

The aim of BCG instillation, besides prophylaxis is tumor destruction. Significant proportions of patients (44%) have residual disease after resection when initially diagnosed as having a T1 disease [84]. Other candidates for BCG tumor eradication include those patients with known residual disease for whom primary or secondary

resections are not possible. This strategy is useful for small papillary tumors and it is recommended that these tumors be no larger than 2 cm. BCG can destroy a large number of residual tumors as long as they fall under this size restriction. However, the category or stage of patients that receive the most benefit from BCG therapy are those patients with primary CIS bladder cancer or those that experience multiple recurrence of superficial papillary TCC [9, 85]. The need or role for maintenance BCG has yet to be defined. Efficacy of BCG has been tested compared to other common forms of TCC treatment. BCG provides superior effects on tumor recurrence compared to chemotherapy (thiotepa or doxorubicin) [26, 86].

Other Immunotherapies

There are many methods of modulating the immune system. Other options for stimulating the immune system can be less toxic compared to BCG but none have shown the efficacy of BCG. Natural chemicals (cytokines) used by the immune system to activate T cells and directly destroy aberrant cells or pathogens are prime candidates to be used for immune therapy. Some of these therapeutic options have been investigated for potential use as intravesical or intravenous agents to treat bladder cancer.

Interferon (IFN-α or IFN-β) is a prime anti-cancer candidate. IFN directly affects tumor with an antiproliferative effect and indirectly affect tumors by stimulating the immune system. Cytotoxic T cells and lymphokine-activated killer (LAK) cells are activated by IFN and targeted towards the tumor cells. IFN alone may not have enough anti-tumor properties but used in concert with other immunotherapies may be synergistic [87].

Interleukin-2 (IL-2) is another potential cytokine treatment for superficial bladder cancer. IL-2 is an immune system mediator and it is found in the effluent from the bladder after treatment with BCG [45]. This suggests that IL-2 plays a role in BCG mediated tumor destruction. IL-2 may be synergistic and allow for lower doses and potentially lower side effects when used in combination with BCG for instillation therapy. A clinical trial of intravesical administration of IL-2 alone for the treatment of TCC by Gomella et al produced a 21% overall response rate [88]. Though this response rate is inferior compared to BCG it may provide an option to those patients that do not respond to BCG therapy.

Keyhole-Limpet hemocyanin (KLH) is another immune that has been evaluated for treating superficial bladder cancer. KLH is a natural derived protein of high molecular weight that is capable of inducing an immune response in humans. Unlike IL, KLH is not normally found in human serum. In humans KLH will initiate both a cell-mediated and humoral immune reaction. In a study conducted by Jurinic et al only 14% of treated patients suffered from tumor recurrence and no patients undergoing treatment with KLH suffered from adverse side effects [89]. KLH may have a role in preventing tumor recurrence.

Oncolytic Viruses

Viruses have been related to cancer biology in several different ways. From the initial discovery of oncogenes and proto-oncogenes to recent advances in the oncogenesis theory, including gatekeeper and caretaker genes, molecular oncology research has used viruses. It has long been established that certain viruses have the ability to transform cells in culture. There are multiple ways in which a virus can perturb cancer cell replication. One way is the direct lysis of cancer cells by productive viral infection/replication termed 'oncolysis'. There is the possibility that the selective nature of certain viruses in cell binding could be exploited in targeting cancer therapy.

There are oncolytic viruses already in clinical trials. ONYX-015 is a genetically altered Adenovirus (a common virus used for gene therapy) [90]. This natural virus produces a protein that represses the p53 tumor suppressor gene in host cells, which would normally prevent viral replication. This altered virus does not express this protein and is therefore selective for dividing tumor cells lacking the p53 gene product. In theory

ONYX-015 will only infect and destroy tumor cells. Early studies are still being conducted on this recombinant virus [90].

Another oncolytic virus being tested in the clinical setting is again a modified adenovirus CN706 [91, 92]. This virus has been altered such that it expresses a regulatory gene, which normally represses the expression of prostate specific antigen (PSA). Prostate tumors generally express high levels of PSA. In these recombinant viruses, protein will only be produced in the cellular environment that is synthesizing PSA. In theory this would target active viral infection only to those cells expressing PSA, which would allow for selective lysis of prostate tumor cells. A limitation however is that there is circulating PSA in free and bound forms.

Human herpes virus is another oncolytic virus in clinical trials [93-95]. This virus had to be genetically altered so that it would not harm normal cells. Herpes virus can cause encephalitis in humans, in the recombinant virus these genes were deleted. All the genes the virus required for independent replication within the cell were deleted from the clinical form of herpes simplex G207 [96]. Some tumor cells make large amount of normal and aberrant enzymes that are required for viral replication. These host enzymes allow for the replication of G207 and subsequent replicative lysis. Because so many genes where removed from G207, it is unlikely that random mutation or recombination would produce reconstituted progeny virus, capable of infecting healthy cells [97, 98].

These examples of oncolytic viruses used today in clinical trials all represent recombinant or altered forms of naturally occurring viruses. Parvovirus, a potential oncolytic virus was also considered for clinical use, but failed in early trials. This is believed to have resulted from reduced infectivity or cellular adherence [99]. It has since

been re-engineered in an attempt to enhance infectivity and therefore increase oncolysis [99, 100]. Recent work, by Dr. Lee et al has illustrated the fact that a non-engineered mammalian virus, REO, has specific oncolytic properties [101]. REO has been tested in tissue culture and in severe combined immunodeficient (SCID) mice bearing xenografted tumors. Oncolytics Biotechnology Incorporated, under the direction of Dr. Don Morris is currently initiating human trials with REO in Calgary, Alberta, Canada.

Mammalian REO belongs to the genus Orthoreovirus in the Reoviridae virus family. REO was named after the fact that it is a respiratory enteric orphan virus. The term orphan refers to a virus to which no human disease is caused by infection. The virus monocistronic genome is a dsRNA molecule 27kbp in length. The morphology of REO is icosahedral with no envelope. The virus is generally stable and can resist heat, pH extremes. The virus remains viable in acidic environments as low as pH 3 [102]. This protection contributes to the virus ability to infect cells of the gastrointestinal tract. REO infectivity is increased by proteolysis; such as the enzymatic processing that occurs in the stomach. REO is believed to enter the cell by receptor-mediated endocytosis [101]. The virus attachment protein, a minor outer capsid protein σ1 is located on the vertices of the icosahedron [103]. It has been established that REO recognizes α-sialic acid on the surface of the host cell. The specific proteins recognized by REO are still being researched. Viral binding and internalization alone is not sufficient for active REO infection. Translation of viral proteins is required for infection and cell lysis. Epidermal growth factor receptor (EGFR) is one of the binding proteins recognized by REO and believed to be required for internalization [103]. REO is not an antagonist at the EGFR for epidermal growth factor. Binding of REO to EGFR may actually enhance EGF ligand

binding. Though EGFR allows REO to bind, it is not needed for active infection, REO can bind any α-sialic acid moiety found on many surface proteins.

A secondary mechanism used by REO for cell entry is via intermediate subviral particles through the endosomal pathway. It is also believed that these particles may also enter the cell directly. Subviral particles are created by the digestion of complete particles with proteolytic enzymes, such as chymotrypsin [103].

Once internalized, intact REO or subviral particles are degraded into icosahedral core particles. Viral transcriptase transcribes the 5' capped mRNA which are exported from the icosahedral core. An early viral gene product represses viral transcription; this complicates investigations into the replication of virus within the cell. It is known structurally that new virus particles accumulate in the cell as inclusion bodies before ultimately lysing the host cell [104].

The pioneering research of Dr. Patrick Lee's group has shown that REO will selectively infect and lyse transformed cells. However, binding, internalization, uncoating and early transcription of viral gene products occurs in both normal and transformed mammalian cells. In untransformed cells, the early viral transcripts either directly or indirectly cause the phosphorylation and activation of a 65-kDa RNA dependent protein kinase (PKR), which in turn leads to the phosphorylation of the translation initiation factor eIF-2α [101]. Phosphorylation of the eIF-2α stops further translation and hence replication. Transformed cells with a deletion in the gene encoding the PKR protein are therefore susceptible to REO infection (figure 3). It has been hypothesized that REO oncolysis occurs through the Ras phosphorylation-signaling cascade. The theory proposed by Dr. Lee et al states that Ras or one of its downstream elements prevents or

reverses the phosphorylation of PKR thereby allowing REO protein translation and viral infection. Dr.Lee's group has previously established that the Ras signaling pathway produces inhibitory elements involved in PKR signaling [101].

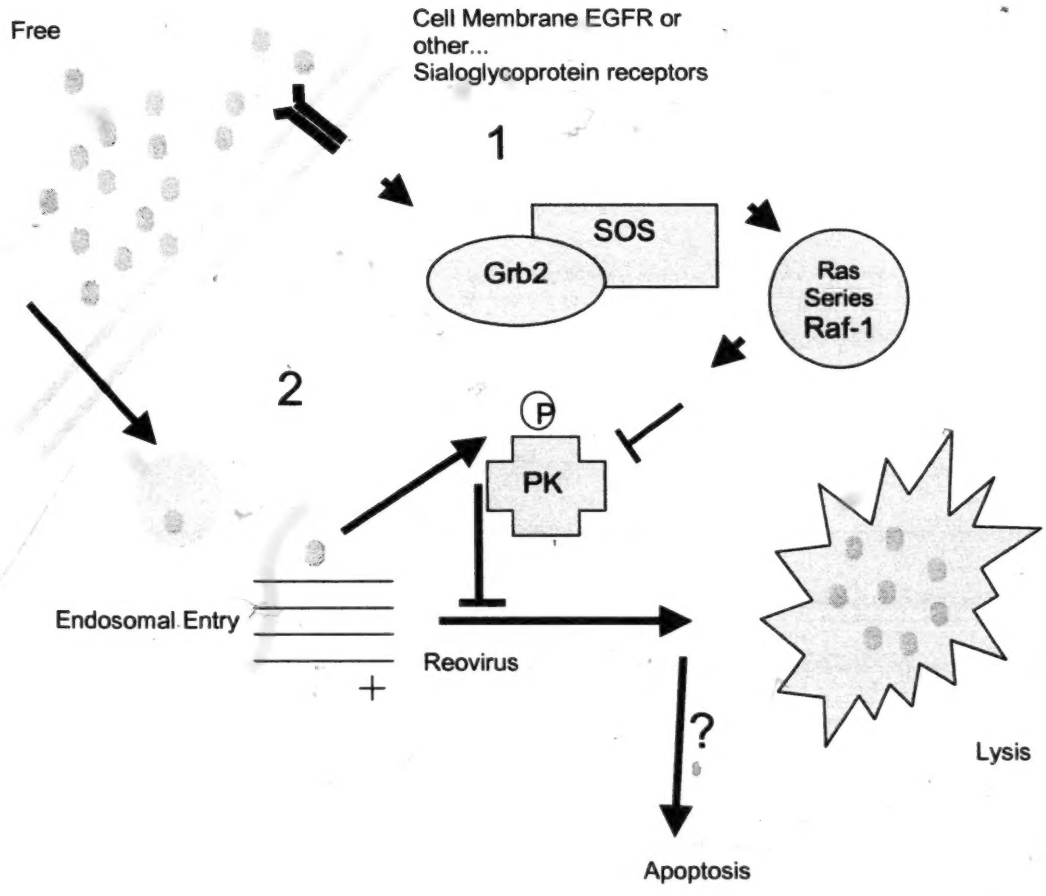


Figure 3. Kinase signaling and the interrelationship with REO oncolysis. Positive arrows indicate progression while blunted (square or round) indicate inhibition. Both receptor mediated cell entry and sub-viral entry are indicated. Apoptosis pathways occur after viral proteins are produced.

The Ras families of oncogenes have long been known to be associated with many human tumors. Approximately 30% of all human tumors express some form of activated ras ongogene [105, 106]. The fact that one of the three common Ras point mutations in codons 12, 13, or 61 (H-ras, K-ras, and N-ras respectively) are found in many tumor types and that the EGF pathway is constitutively over expressed in many tumors supports further exploration into the selective targeting and mechanism of REO oncolysis. As many as 17% of bladder carcinomas express the H-ras phenotype [107, 108]. This tumor cell specific phenotype may allow REO oncolysis to be highly selective for treatment of these transformed cells

Two mechanisms of oncolysis have been elucidated. One via direct replicative lysis and the other by apoptotic pathways [109, 110]. Oncolysis occurring by selective apoptosis in tumor cells would not affect adjacent healthy cells. The actual mechanisms of induction of the apoptotic pathway are currently an area of intense research. The specific details however have yet to be defined. Common induction pathways of apoptosis include activation of the gatekeeper genes, which basically induce cell suicide for the benefit of the whole tissue and ultimately the organism. The immune system is a common activator of apoptosis in cells. This can be done during development of specific immune cells, but also during times of immune system activation such as viral infection and inflammation. In general anything that stresses a cell can lead to apoptosis. Stress can be internally derived as a result of a faulty genome or externally derived as a result of foreign insult.

Viral induced apoptosis has proven to be one of the more complicated apoptotic pathways to resolve. This is understandable when one considers the many different protein agonist/receptor relationships that are involved in viral infection. Research has shown that interferon (IFN) is a common defense to viral infection used by the immune system and is also implicated in apoptosis pathway induction [25, 110]. This is especially true when interferon and RNA is introduced to cells in culture. This mimics the situation seen with REO, as the viral genome is dsRNA and during infection, the activated immune system will release a cytokine profile including members of the interferon family. PKR is dependent on RNA for activation and is induced by the presence of interferon [111]. Interferon has been used in combination with natural viral oncolysis in the experimental treatment of breast cancer [112]. The strategy involves producing a recombinant adenovirus that encodes for the IFN. The exact mechanistic role interferon plays in enhancing PKR is not well understood. Normal cells that have been altered genetically so there is no production of the PKR protein are resistant to several apoptotic signals. Stimuli including dsRNA, tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) have no apoptosis inducing effect on these altered cells. In the apoptotic-signaling pathway PKR is a mediator, interfering with the normal transcription of this gene some how alters the signal for programmed cell death.

As tumors replicate and accumulate genetic errors such as signaling protein mutations, viral oncolysis may offer the best route of treatment. The chemotherapeutic agent Paclitaxel (Taxol™) for example has reduced cytotoxic effects on tumors expressing the raf-1 kinase phenotype [114]. Therefore these proteins may be involved in mechanisms in which tumors can become drug resistant [114]. It may be that aberrant

expression of the same proteins normally involved in intracellular signaling may predispose the tumor to viral infection and oncolysis. *In vivo* and *in vitro* research has pointed to the over expression of the raf family of cell signaling proteins in many tumor types including bladder tumors.

PROPOSED RESEARCH PROJECT

The background literature review in this thesis covers current bladder cancer diagnosis and treatment theories. The experimental portion of this thesis involves a pre-clinical translation experiment testing a novel oncolytic therapy in superficial bladder cancer model. Dr. Moore's lab has previously established an experimental animal model to test PDT treatments. This model was used to test REO as a treatment for superficial bladder cancer. In tandem, current intravesical therapies were tested and compared to REO. BCG in varying dosing schedules with or without IL-2 was compared to intravesical REO. Animals were randomly assigned into the treatment groups in sufficient number that would allow for statistical analysis. Survival and toxicity was monitored and organs were examined histologically at endpoint. The implanted cells (AY-27) used in the tumor model were tested *in vitro* for susceptibility to REO infection and oncolysis. Cells were tested quantitatively using an MTT cytotoxicity assay and qualitatively using immunohistochemistry. Selective oncolysis was examined by using co-cultures of TCC and normal fibroblast cells. *In vitro* response to REO using varied time courses and infection concentrations was monitored. The working hypothesis governing this research was that REO would reduce tumor load and therefore affect animal morbidity and mortality. The hypothesis was generated on the foundation that novel intravesical therapies have a place in current TCC management strategies and that REO has potential as an intravesical therapy to be used in patients refractory to current treatments.

MATERIALS AND METHODS

Preparation and Culture of Rat TCC

AY-27 rat TCC was previously characterized for use in an animal model of superficial bladder cancer in Dr. Moore's research laboratory [115]. Cells are routinely maintained in adherent monolayer (RPMI-1640 medium Gibco-BRL, 10% heat inactivated fetal bovine serum in 37°C humidified 5% CO_2) at low passage to preserve genotype. The cells are regularly passaged heterotopically to Fischer F344 rats to maintain low grade TCC stage and to ensure no pathogen contamination (either bacteria, fungal or mycoplasma). The AY-27 cell line was originally induced in the bladders of Fischer F344 research rats by using the FANFT carcinogen, and was generously provided previously to our lab by Dr. Steve Selman from the Medical College of Ohio, Toledo. At time of their production and harvest Dr. Selman characterized them as being primary superficial TCC in character [115]. This cell line was further characterized for its TCC phenotype on arrival to the Moore lab and subclones were selected that would form tumors when implanted intravesically. These cells were expanded in culture and stored in frozen aliquots in liquid. These cells were subcultured using standard mild trypsinization. For each subculture the AY-27 cells were washed 3 times in warm (37°C) phosphate buffered saline (PBS) and then incubated with 0.01% trypsin EDTA for 5-7 minutes in a humidified incubator. Cells were kept for implantation with a generation number of less than 20 to preserve TCC genotype.

In Vitro Kinase Analysis

AY-27 and MGHU3 cytosolic protein fractions were isolated by incubation with a protein lysis buffer (50 mM Tris HCl pH 7, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet

P-40, 2 mM sodium, orthovanadate pH 10, 10 ug/ml leupeptin, 10 um/ml aprotinin, 1 mM PMSF) after regular cell washing and trypsinization (as previously described). Raf-1 was isolated from protein fractions by immunoprecipitation using an agarose conjugated mAb (Santa Cruz Biotechnology C-12 cat # sc-133 AC) for an overnight incubation period at 4°C.

The Pierce Colorimetric PKC Assay Kit, SpinZyme™ Format protein assay was used to determine functional levels of isolated protein kinase. All samples were read with a Bio Rad Microplate Reader Benchmark series. Kinase activity was calculated relative to a standard curve created using purified protein kinase C (Pierce product # 29536).

Western Immunoblotting

Protein was isolated from cultured TCC after cells were trypsinized (0.1% trypsin EDTA) and re-suspended in lysis buffer (10 mM HEPES pH 7.2, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, PMSF 0.2 M 50X stock). Aliquots then underwent 3 freeze thaw cycles in liquid nitrogen before being centrifuged gently to pellet nuclei. Protein quantification was then done with a standard Bio-RAD Bradford assays (Bio-Rad DC Protein Assay 500-016). Western immunoblotting was then performed on isolated samples following standard 2-dimensional polyacrylamide gel electrophoresis techniques [116]. A primary antibody directed against raf-1 was used (Raf-1 C-20 Santa Cruz Biotechnology SC-227 Lot#092 rabbit polyclonal antibody). Western immunoblotting was used to determine the presence of Raf kinases to compliment quantitative kinase assays, which ensured function. Maximum amounts of whole cell protein isolates were loaded into gels to detect any amounts of kinase protein present.

MTT Cytotoxicity Analysis

Adherent cell lines were assayed for *in vitro* oncolytic response to REO. A Standard 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) cytotoxicity assay was used [117]. Cells were grown in culture to sub-confluent levels in a 75 mm culture flask. At this point the cells were trypsinized and washed in PBS before being counted using a coulter counter (Coulter Electronics, Canada). Cells were plated on a 96 well plate (10 000 cells / well) on day 1 in their required growth media supplemented with fetal bovine serum (10-20%). Selected cells were initially sub-confluent thus ensuring log-phase growth (low generation number). After a minimum of 10 hours incubation (doubling time 24 hours) growth media was removed and virus of various titres was added in either serum free media, serum supplemented media or PBS for varying incubation times. The cells were then incubated along with virus (37°C 10% CO_2). All media was removed and MTT was added (10 ug/ml) in a volume of 50 ul (phenol free media). In some cases, the plates were washed with PBS to remove residual media color (when phenol free media was not used). Cells were incubated with MTT and virus (37°C 10% CO_2) for 4 – 6 hours in the dark. Resulting formazan crystals (from MTT metabolism by mitochondria) were read with a spectrophotometer at 580 nm read 688 nm reference (Bio-Rad Microplate Reader Benchmark).

Immunohistochemistry

Cells were first cultured in 75 mm flasks and allowed to grow to sub-confluent levels (80-90%). Once gently trypsinized and washed with PBS, cells were grown on positively charged pre-sterilized microscope slides a humidified incubator (37°C 10% CO_2). Slides were placed in a humidified 5% CO_2 incubator in large Petri dishes covered

but not sealed. Once cells were established and confirmed using upright microscopy to be growing adherent to slides, REO of varying titers was added to the dishes. At this point, since Petri dishes contained slides that were open, the experiment was transferred to a REO positive incubator (37°C 10% CO_2). Control slides not infected with REO remained in the standard lab virus-free incubator. Slides were exposed to virus for varying time courses (0-6 days). All slides were then dehydrated in ethanol (50%, 70%, 95%) and fixed using 0.1% formalin PBS using fixation times between 1 to 5 minutes. The primary polyclonal (rabbit anti-REO) antibody was graciously supplied by Dr. Patrick Lee. Prior work by Dr. Lee established that this polyclonal antibody binds to the outer REO capsid proteins. A standard ABC™ immunohistochemistry DAB kit was used to detect intracellular REO capsid proteins.

Multicellular Spheroid Analysis

Dr. Ruhangiz Taghi-Kilani (Dr. Moore's research assistant) conducted the multicellular spheroid studies with my consultation and assistance. I helped develop and learned how to manipulate all protocols. Growing a single cell type on a semi-solid agar overlay generated MCS cultures. A standard agar overlay protocol was followed [118]. Monolayer adherent cells were detached using gentle trypsinization (0.01% trypsin EDTA – GIBCO) and centrifuged before being counted using a coulter counter (Coulter Electronics of Canada Ltd.) Cells were plated in 96-well dishes (3,000 – 10,000 cells/well) on a 1% agarose layer. After 3 days of incubation to allow for settling and growth, MCS were formed. Cells were then transferred to co-cultured wells again with a 1% agarose base. At this point MCS co-cultures could interact and be exposed to REO of varying concentrations ($5x10^7$ PFU – $1.7x10^8$ PFU). After varying REO incubation times

MCS co-cultures could be visualized with a ZIESS Axioplan II inverted microscope equipped with a digital CCD camera to generate images.

Specific cells in MCS co-culture could be detected using vital dye and IHC. Cultured MCS exposed to REO could be fixed using 10% neutral buffered formalin, paraffin embedded and sectioned. Cell populations could be differentiated using IHC techniques previously described in this thesis. Primary antibodies to detect TCC (cytokeratin 13 clone CAM 5.2 BD Canada) and fibroblasts (Vimentin DAKO) were used.

Vital dye staining and confocal imaging was performed using live dead assays on MCS co-cultures. The vital dye Syto 16/PI was used in conjunction with confocal laser microscopy at varying time points. MCS studies allowed for visualization of REO effects on both normal (fibroblast) and cancerous (MGHU3) cells grown together in close proximity. MCS co-cultures were the last *in vitro* assay to be used prior to moving to animal experiments to test the efficacy and side effect profile of REO as a treatment for TCC.

Animals

Female syngeneic Fischer F344 rats (n>100) were used for tumor implantation and randomly assigned to an experimental group (n=10). Animals were either purchased from Charles River Laboratories or bred locally in a clean conventional (biohazard level 2) vivarium (Cross Cancer Institute). Prior to being used for experimentation all animals were allowed to acclimatize to the vivarium facility. Each animal was implanted at a minimum of 6 weeks of age and/or body weight of 0.150 kg (figure 4). Implants were performed as originally established by Dr. Xiao [115]. This is a reliable and reproducible

TCC animal model designed to use transplanted cells originally transformed and selected for a pure TCC phenotype when implanted into syngeneic animals. Prior to implantation all animals were visually in good health. All small animal procedures were carried out in accordance with guidelines from Canadian Council on Animal Care (CCAC) and approved by an in house animal ethics committee. Sterile surgical technique was used for all procedures involving instrumentation (catheterization) of the animals (figure 5).

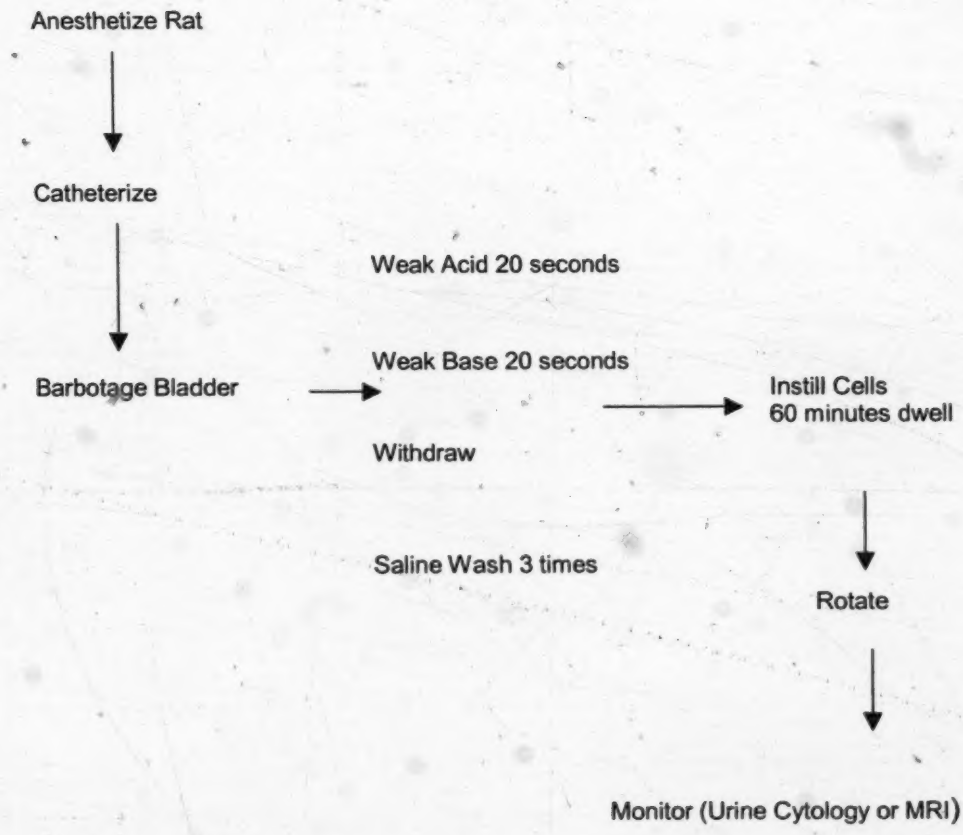
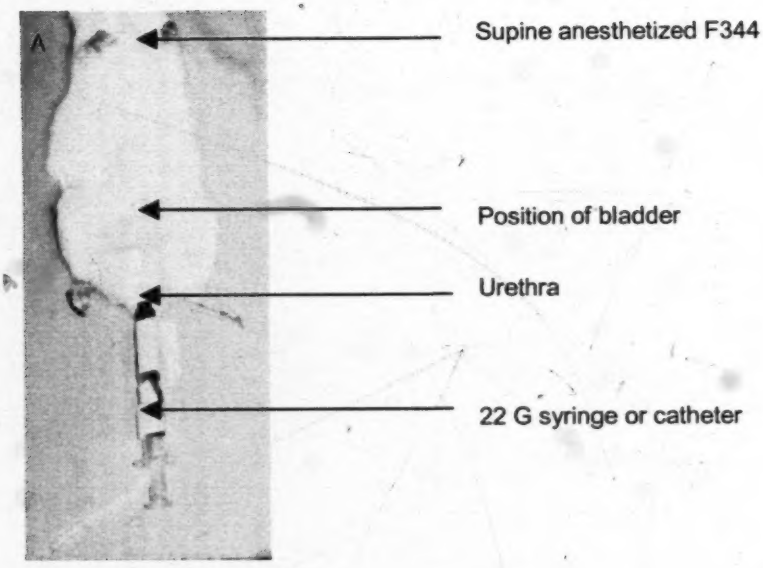


Figure 4. Implantation regime for a transplantable, orthotopic superficial bladder cancer tumor model



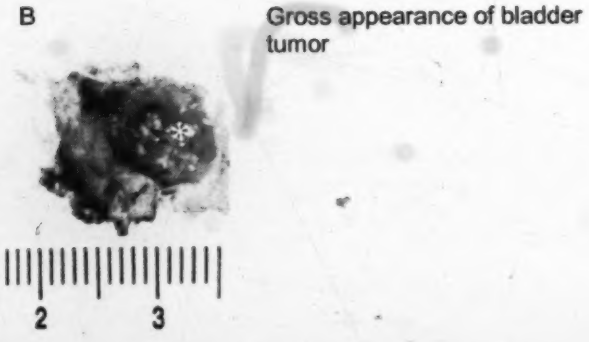


Figure 5. A. Experimental setup for tumor cell transplantation and intravesical therapy. B. Gross features of a typical mid to late stage papillary bladder tumor in an untreated rat (~1 cm all dimensions).

Animals that were shipped to the University of Alberta Health Sciences Laboratory Animal Services (HSLAS) 1 week after tumor implant by an internal animal transport system. Once at HSLAS (biohazard level 3), animals were allowed to re-acclimatize and were monitored at least 3 days for signs of distress prior to initiating treatment. With the assistance of lab animal services the animals were monitored daily for signs of distress while in the level 3 facilities.

This animal model system is very cost effective and animals are large enough to allow for surgical manipulations such as catheterization of the urethra to be possible without risk to the animal. This system is now used around the world to study TCC in a reproducible fashion.

Tumor Cell Implantation

Exponentially growing rat TCC AY-27 cells ($2x10^6$) in 500 ul of serum free media were seeded intravesically (within bladder) by urethral catheterization (18 Gauge angiocatheter). Animals were anesthetized with a combination of intraperitoneal (i.p.) ketamine (90 mg/kg) and xylazine (10 mg/kg) for each instillation, including tumor cell implantation (figure 4). If prolongation of surgical plane anesthesia was required, additional ketamine and xylazine were administered i.p. at a half dose and then quarter dose. The day of tumor implant was considered to be day 0 and intravesical treatments commenced at day 10. The tumor implant followed the exact protocol designed by Xiao *et al* [115]. The actual day of implantation is considered day 0 and day 10 is considered to be early tumor growth in the bladder as demonstrated in prior studies.

Urine Cytology

In previous studies, animals were monitored using magnetic resonance imaging (MRI). This model has proven reproducible and reliable with 90% tumor establishment [115]. MRI monitoring of tumor has been replaced in this study in part with standard urine collection and cytology. This was a late addition to our animal model originally described by Xiao *et al.*

For the first time in the history of the transplantable orthotopic bladder tumor model used in Dr. Moore's lab, urine cytology was examined. The protocol was used later in the project, so not all animals were tested. This was a pilot study and did not affect the outcome of the main survival/toxicity study of REO. Urine was collected at days 0, 10 and prior to sacrifice. Approximately 0.5 ml of urine was collected through the catheter used either for tumor cell implant or intravesical therapy. Not all catheterizations resulted in collectable urine samples. In such cases, a small amount (<0.5 ml) of normal saline was introduced as a wash into the bladder and analyzed for cytology. In cases where there were large amounts of hematuria, samples were diluted with normal saline prior to analysis. A cytotechnologist was consulted on the analysis of stained (Papinicolau) cytology slides (table 3).

Cell morphology especially membrane architecture was a key diagnostic characteristic. Specimens were reported as being benign, inflamed, suspicious, or outright malignant. All slides were examined by a cytologist and only identified by animal number. In cases where cell density was too low, a low cellularity report was documented. In some instances when hematuria was frank, samples had to be diluted with saline to clear up RBCs from the field of view for proper diagnostics. A potential

option for future studies would be a miniature fiberoptic cystoscope designed to view rat bladders for tumors. However, at present, such a tool does not exist.

Intravesical Therapy

BCG was obtained from Aventis Pharmaceuticals (formerly Pasteur-Merieux Connaught Canada). Prior to shipment it was quantified for colony forming unit (CFU) quality and treatment aliquots were based on these functional calculations. BCG was used in aliquots and never stored after use (constituted in sterile 0.9% saline). All lyophilized BCG aliquots were kept at 4°C prior to instillation into the bladder. BCG experiments were done at the CCI clean conventional animal vivarium under level 2 biosafety precautions following CCAC guidelines.

Animals were randomly divided into treatment groups (n=10) (table 4). They were assigned to low, medium, or high levels of BCG twice weekly for 3 weeks. One group (n=10) was created having medium dose BCG administered once weekly for 6 weeks. This dose schedule was included in case the twice weekly instillations produced an anergic response overwhelming the immune system therefore promoting tumor growth .A Sham control group (n=10) was only given saline. BCG dosing with or without immune stimulants (IL-2) was designed to test dose responses and side effect profiles. Limiting BCG dosing and instillation frequency may reduce toxicity.

Table 4. A. BCG intravesical treatment groups. B. REO treatment groups. All treatments start at day 10 post implant.

A.

Treatment Group	BCG (CFU/ml)	Volume Instilled	Schedule	Number	Notes
Low BCG	$5x10^5$	0.5 ml	2x / week / 3 weeks	10	
Medium BCG	$5x10^6$	0.5 ml	2x / week / 3 weeks	10	
Medium BCG	$5x10^6$	0.5 ml	1x / week / 6 weeks	10	
High BCG	$5x10^7$	0.5 ml	2x / week / 3 weeks	10	
IL-2/BCG	$5x10^5$	0.5 ml	2x / week / 3 weeks	10	$5x10^5$ units rIL-2
Saline	0	0.5 ml	2x / week / 3 weeks	10	0.9% saline

B.

Treatment Group	Reovirus (PFU/ml)	Volume Instilled	Schedule	Number	Notes
Low REO	$5x10^5$	0.5 ml	2x / week / 3 weeks	10	
Medium REO	$5x10^6$	0.5 ml	2x / week / 3 weeks	10	
High REO	$5x10^7$	0.5 ml	2x / week / 3 weeks	10	
Saline	0	0.5 ml	2x / week / 3 weeks	10	0.9% saline

Sham animals were compared to previous intravesical PDT studies to validate the reproducibility of the transplantable model.

Intravesical therapy setup was identical to tumor transplant but no pre-treatment of the bladder was done. This ensured that urothelium was kept in state consistent with the natural history of bladder cancer. BCG was instilled in 0.9% sterile saline in a volume of 0.5 ml. This volume in a rat bladder provides an even fill with no ureteral reflux or high pressure levels [115]. The instillation lasted 20 minutes with a 90° whole body rotation done every 5 minutes. After this time period the animals were allowed to recover in their cages. When urine was collected for cytology (at day 10 for implant monitoring) it was done before instillation of BCG to ensure no mycobacterium clouded the cytology reading.

Once the animals completed the treatment regime, they were observed for at least 100 days for signs and symptoms (table 2) of bladder cancer (decreased body weight, and hematuria). Animal stress (piloerection, ocular staining and lordosis) can be early indicators of cancer in rats and was also monitored by animal technicians. After the observation period all animals were euthanized using either pentobarbital overdose or CO_2 inhalation and subjected to necropsy. The bladders and kidneys were excised for histological analysis (hematoxylin and eosin staining). Any other organ that appeared abnormal was also excised and studied microscopically. Inguinal lymph nodes were also examined for signs of inflammation or systemic infection. If any animal became severely distressed prior to the 100 days they were euthanized and underwent necropsy.

Mammalian reovirus serotype 3

REO was supplied by Dr. Patrick Lee's research lab. Aliquots were used within 1 to 2 weeks upon arrival to the lab. All samples were purified and infectious units were calculated prior to delivery to our lab. Plaque forming units (PFU) were calculated using good laboratory practices (GLP) prior to delivery. REO was diluted for intravesical therapy in sterile 0.9% normal saline.

REO was administered to the animals intravesically using the same experimental protocol as BCG therapy with the exception of biocontainment level (level 3 versus level 2). Dosing in REO treated groups paralleled BCG groups (table 4b). All REO treated animals were housed in a dedicated biohazard level 3 facility (HSLAS). No other animals were housed in the same airspace as the REO groups.

Recombinant Interleukin-2 (rIL-2) was obtained from the Cross Cancer Institute Pharmacy. Interleukin activity (units/ml) was calculated prior to delivery to the lab. All rIL-2 was kept sterile and at –80°C. Aliquots were thawed prior to use and diluted if necessary in sterile saline before being mixed with BCG. Mixed rIL-2/BCG was used immediately for treatment.

Histological and Statistical Analysis

A pathologist was consulted for all histology (randomized-blinded). Slides were only labeled with animal ID numbers and not treatment groups. Other organs that appeared abnormal at time of necropsy were also removed and prepared for histology. Features examined in each slide included; grade, stage, mitotic figures, N/C ratio, lymphocyte infiltration, hemorrhage, and necrosis. Final slide diagnosis was discussed but ultimately confirmed by the pathologist.

Statistics were computed using SAS v6 and Visual Basic testing of survival groups included an initial z-test with log-rank comparisons made to detect inter-group differences. Individual groups were compared relative to each other using Chi squared analysis and a student's t-test was used to calculate p values. A p value of less than 0.05 was considered to be significant for paired group comparisons.

RESULTS

In Vitro Kinase Analysis

The colorimetric assay was able to detect and quantify using spectrophotometry and fluorometry, the amount of functional protein kinase present. The actual kinase detected was determined by the monoclonal antibody used to precipitate cRaf-1 from complete whole cell lysates. Cell signaling kinases, including cRaf-1, are all cytosolic proteins. Standard curves were generated using purified protein kinase C as an indicator of kinase activity (positive control). Using this standard curve generated from the positive control, functional units of kinase activity could be assigned to protein isolates (figure 6). Consistently, kinase was detected and functional in cultured TCC (AY-27, MGHU3). Rat TCC showed higher levels of both MAPK and cRaf-1 activity. In both species TCC cRaf-1 had a higher activity compared with MAPK. The MGHU3 human TCC line had approximately $6x10^4$ U of kinase activity (both MAPK and cRaf-1), and cRaf-1 was slightly higher. The rat AY-27 TCC line had approximately $7x10^4$ U of kinase activity (both MAPK and cRaf-1), again cRaf-1 was elevated (figure 6). Equal numbers of cells ($1x10^6$) were used for protein analysis to prevent quantitative differences being due to cell number.

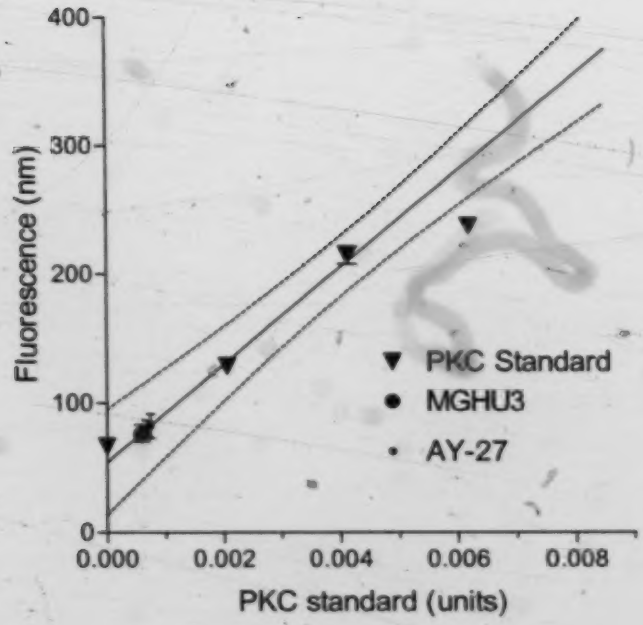


Figure 6. Protein kinase activity determined using a functional assay. Units of kinase activity were calculated by using a standard PKC control set. Standard error isobars are included.

In Vitro Reovirus Cytotoxicity

Cells exposed to REO did respond and demonstrated varying degrees of oncolysis. Qualitatively, signs of cell death could be seen with the light microscope when checking for cell response prior to MTT assay. These signs included non-attached cells and cellular debris when compared with non-infected controls. Other signs of TCC death seen with light microscopy included pyknotic nuclei and cell shrinking. The AY-27 cell line responded to REO in an initial inoculate dose dependent manner. There was decreasing cell survivorship as initial virus titer was increased. Virus titre in 3 to 7 day exposure times varied from $1x10^2$ to $3x10^7$ PFU for initial cell densities of $1x10^4$ per microtitre plate well (MOI 0.01 – 3000). At these infection levels survivorship dropped to less than 5% compared to non-infected controls, translating to more than 2 logs of cell killing (figure 7). Other bladder cancer cell lines were examined, including human cell lines (MGHU3). This was done to support the clinical application of REO therapy. MGHU3 was not as susceptible to REO oncolysis as AY-27, but was the only human line tested (figure 8). To correlate cell killing to pre-requisite protein expression western immunoblots were done in parallel. The results (figure 8) indicated that AY-27, which was susceptible to REO at low levels of exposure, expressed high amounts of raf-1 protein relative to L929 positive controls and NIH 3T3 negative controls (figure 8b). Relative amounts of protein loaded into the polyacrylamide gels varied from 9 – 70 μg. Direct comparisons of protein amounts determined via western blotting were not done. Quantitative assays were already performed at this point and western blotting was only used in this experiment as a method of determining if any kinase could be detected (functional or non-functional) in maximal amounts of whole cell protein isolates. Exact

amounts of protein loaded were; L929 67.5 µg, MGHU3 15 µg, RT112 9 µg, NIH 3T3 7.5 µg and AY-27 15 µg. Future work could use western blotting to compare amounts of kinase in each cell lines tested. Also housekeeping proteins could be checked for to control gel loading consistency between isolated samples.

Up to 72-hour exposure times of REO quantifying multiplicity of infection (MOI), or infectious virus per cell, ranging from 100 to 500 showed only slight decreases in survival with increasing MOI (figure 9). There was a trend for more cell killing to occur with higher virus titers with this limited exposure time. Controls grew well when not exposed to REO and viable cells were detected both microscopically and with the MTT colorimetric assay.

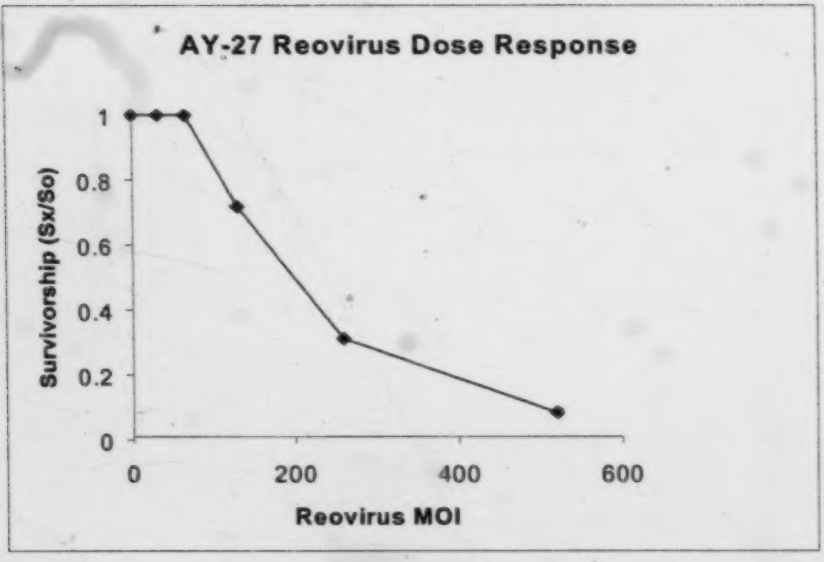
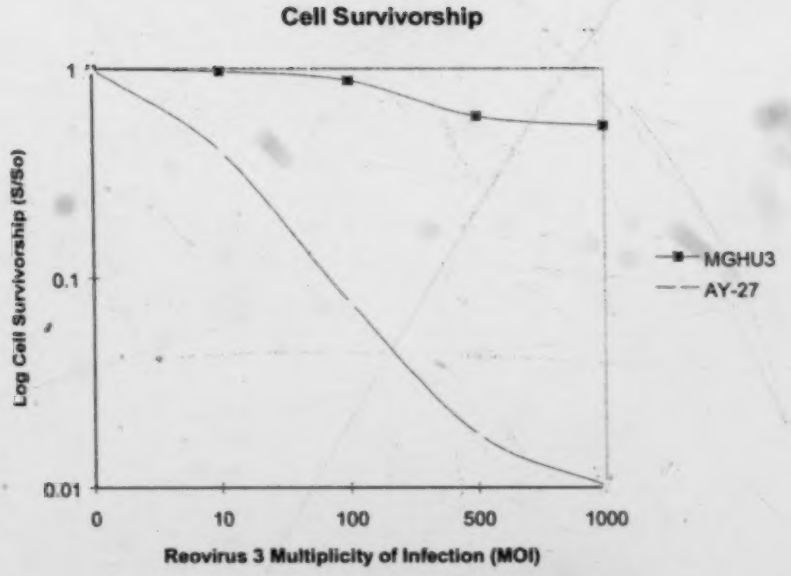


Figure 7. AY-27 TCC survivorship tested using an MTT cytotoxicity assay. The cells were exposed to increasing REO concentrations for a 3 day incubation period.

A



B

Figure 8. A. TCC cell survivorship measure by an MTT cytotoxicity assay to varying levels of REO infection (MOI) using a 3 day exposure. Bladder carcinoma lines examined were AY-27 (rat) and MGHU3 (human). B. Complimentary western blot analysis of cell lines for raf-1 protein expression, a possible contributing factor to REO oncolysis. Loaded amounts of protein ranged from 15-70 μg.

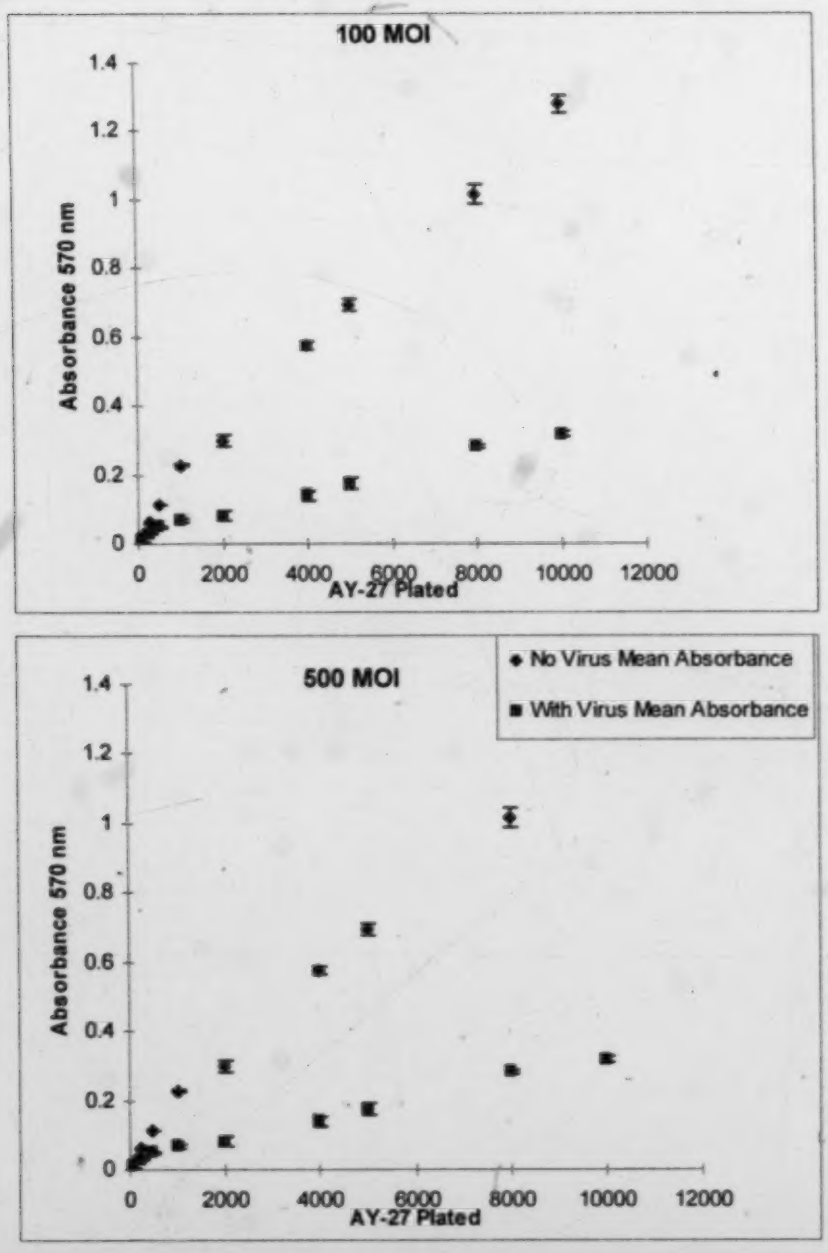


Figure 9. Dose response for varying levels of REO using AY-27 TCC using an MTT absorbency assay (measure 570 nm and reference 680nm). Both virus exposed and negative controls (non-virus exposed) are compared.

Immunohistochemistry

Cells examined using IHC were exposed to REO ($5x10^5$ PFU) for varying amounts of time (1 – 7 days) to allow for REO protein expression. Cells exposed to REO and stained using IHC directed towards outer viral capsid proteins showed increased amounts (subjectively) as exposure times increased. For the AY-27 rat TCC cell line, results were consistent with MTT cytotoxicity assays. Increases with viral capsid protein correlated with increased levels of oncolysis. The quality of IHC images however was poor, even after multiple attempts. Levels of intracellular staining were not intense, but still evident. Staining was cytoplasmic, indicating that viral protein translation took place outside of the nucleus where mRNA has access to cellular translational components. Staining in AY-27 cells became dark and intense (dark brown chromogen) with increased exposure times (figure 10). Cells showed visual signs of cell death (light microscopy) after 2 days of REO exposure. Cellular N/C ratios decreased as exposure times increased, as did cell to cell adhesion contacts normally seen in healthy adherent monolayer growth. Fragmented cells were evident and increased significantly by day 5 and 7. As increases in fragmented and lysed cells accumulated so did extra cellular debris products, heavily stained by dark IHC chromagen reactions, possibly representing intracellular inclusion bodies.

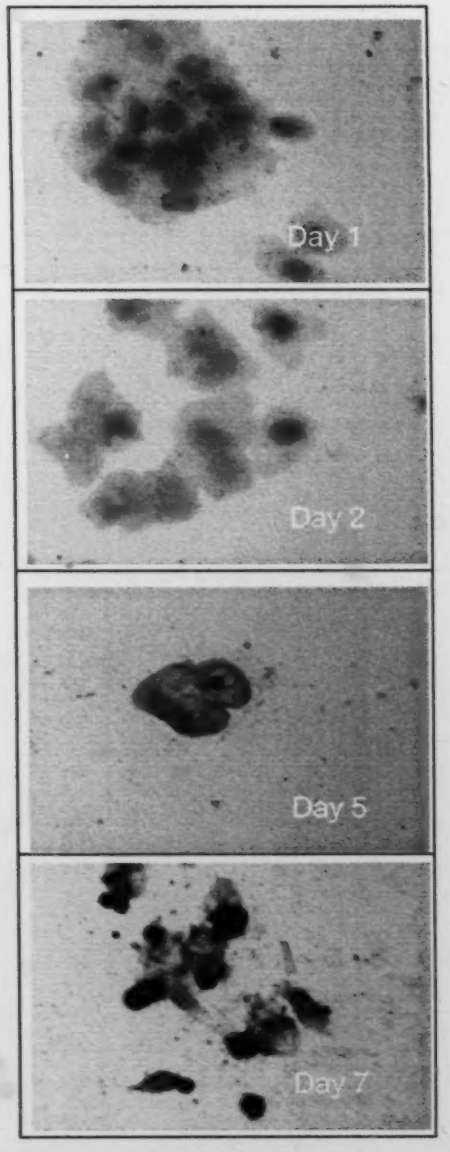


Figure 10. Immunohistochemistry to detect REO particles in AY-27 TCC. Exposure times are indicated in each panel up to one week. An initial dose or REO $5x10^7$ PFU was used to parallel the high dose used in *in vivo* experiments. Staining was not optimal and quantification could not be performed. Light microscopy did reveal normal contacts at day 1, malignant cells would break up and disperse as early as day 3, massive cell death by day 5 with evidence of apoptosis and complete cell destruction of monolayers by day 7 (as indicated in the figure).

MCS Co-Culture TCC REO Assay

Human TCC (MGHU3) and human fibroblasts could be cultured together and individually detected using IHC (Figure 11a). TCC stained cytokeratin 13 positive would normally surround the fibroblast cells completely, using them as a base of attachment (figure 11a). Fibroblast cells stained vimentin positive using IHC were always localized to the center of the MCS co-culture (figure 11a). Increasing amounts REO exposure times resulted in increased peripheral cell death (figure 11b) where TCC is know to grown (figure 11a). The central core of the MCS co-culture was intact and viable even after 14 days exposure (figure 11b). This was not due to limited penetration as pure TCC spheroid cultures were totally ablated and pure fibroblast spheroids were undamaged.

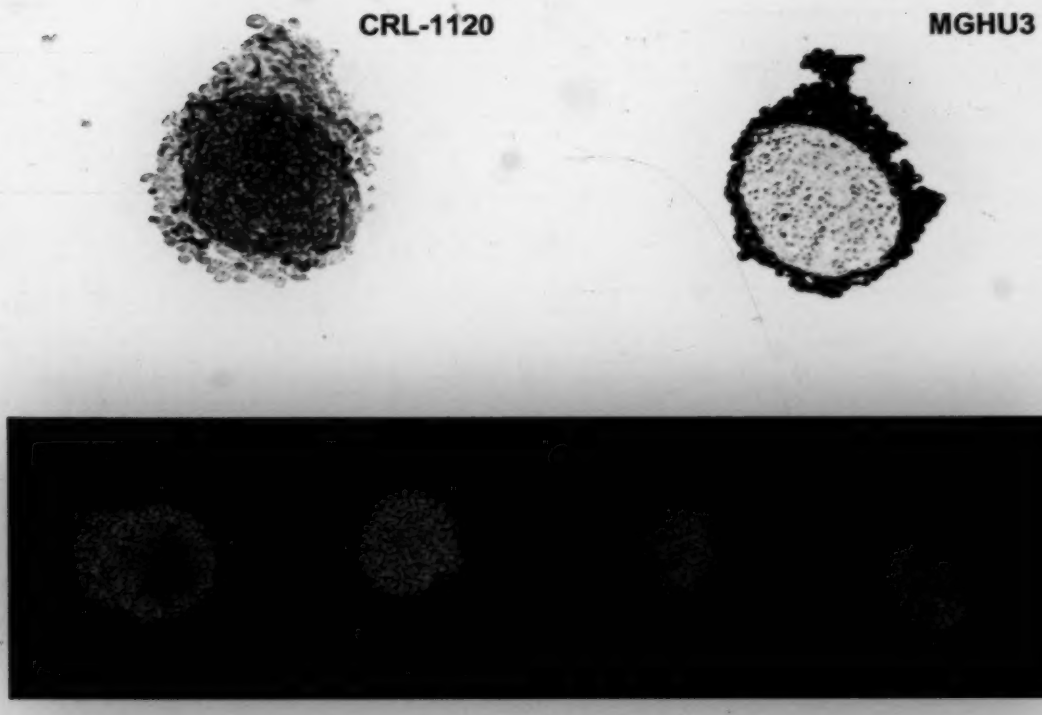


Figure 11. A. IHC detection of TCC (MGHU3) and fibroblast cells (CRL-1120) in a MCS co-culture system. B. Selective viral oncolysis indicated by vital dye staining of MCS co-culture. Peripheral MGHU3 are selectively destroyed at increasing time points (indicated in days) evident by red staining. Central CRL-1120 are alive at each time point evident by green staining.

Transplantable Orthotopic Bladder Tumor Model Implantation

To establish the reproducibility of the bladder tumor model in my hands, a small initial pilot implant group was completed consisting of four animals. Each animal was implanted as outlined and then submitted for necropsy after a minimum tumor growth period of 10 days. All animals were tumor positive being confirmed by gross dissection (100% tumor take). This group was used as a training group to establish surgical technique and lab monitoring skills. Early signs of rat bladder cancer were evident 7-10 days after implantation (hematuria evident from staining of cage bedding). Having successfully achieved a complete tumor take (n=4) in a pilot group, the experiment continued to include the random assignment by lottery drawing of Sham treated (saline) and experiment groups (intravesical therapeutics).

Urine cytology was developed and tested as an add-on after the initiation of the *in vivo* experiment. Prior to this, historic tumor takes of 90% were considered for statistical analysis (n=10 per group therefore a maximum of 1 implant per could might fail). Urine cytology was not very accurate in detecting malignant cells in either urine samples or bladder washes (0.9% normal saline solution), confirmed at experimental end point (necropsy). Cytology had a success rate of detecting tumor positive animals by day 10 of 48% (12/25) and cytological cures by day 60 were determined in 32% of animals (8/25). An initial three animals were monitored for urine cytology in the pilot project and later confirmed at necropsy (early cancer related mortality) to be tumor positive in the bladder (figure 12). Cellular features of TCC were evident in bladder washes. These include increased cell number, abnormal cell morphology, increased nuclear to cytoplasmic (N/C) ratios, the presence of a nucleolus and other histological signs of malignancy

(ruffled cell borders, etc). A clinical cytologist in a randomized-blinded fashion analyzed each cytology sample for all animals and consulted on cytological features.

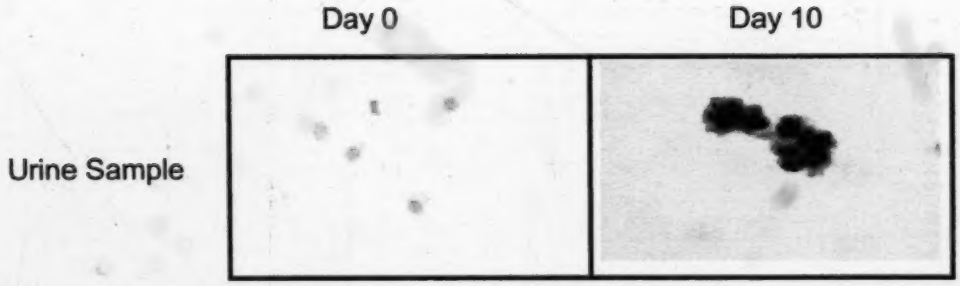


Figure 12. Typical urine cytology results for the AY-27 TCC animal model. Urine collection times are indicated for each implanted animal. Classic signs of positive cytology are evident by day 10 (increased cell number, N/C ratios, malignant cell clustering).

Animal Studies

BCG Intravesical Therapy (Dose Response Study)

BCG was administered at three dosing levels, being low, medium and high. ($5x10^5$ – $5x10^7$). Another BCG group included in the experiment was given at a half frequency relative to other groups. Normally groups received BCG twice per week for three weeks. The half frequency BCG group received BCG once weekly for six weeks. The total number of instillations for each animal was six over a 3 to 6 week period. Sham animals never survived past day 85, being either sacrificed for humane reasons (extreme animal distress) or obvious urological complications (obstruction or uropathy) from bladder cancer (figure 13). The majority (70%) of half frequency dosed BCG animals reached endpoint by day 40, which is extremely early considering historical PDT survivorship studies. These animals also experience the most observable frank complications. The remaining BCG treated animals all showed increased survival relative to the half frequency treated animals or the Sham controls. The highest level of survivorship was demonstrated in the medium dose BCG group that had 45% group survival at day 100. This survival time (day 100 and beyond) was considered a long term survivor (LTS) and deemed cured.

The IL-2 was mixed directly into BCG aliquots prior to instillation in the BCG/IL-2 treatment group. IL-2 mixed well with BCG in saline. IL-2 did impart a survival advantage (figure 12) relative to the low dose BCG counterpart (same dose without IL-2). There were no obvious differences in side effects in the IL-2 group compared with any other BCG group.

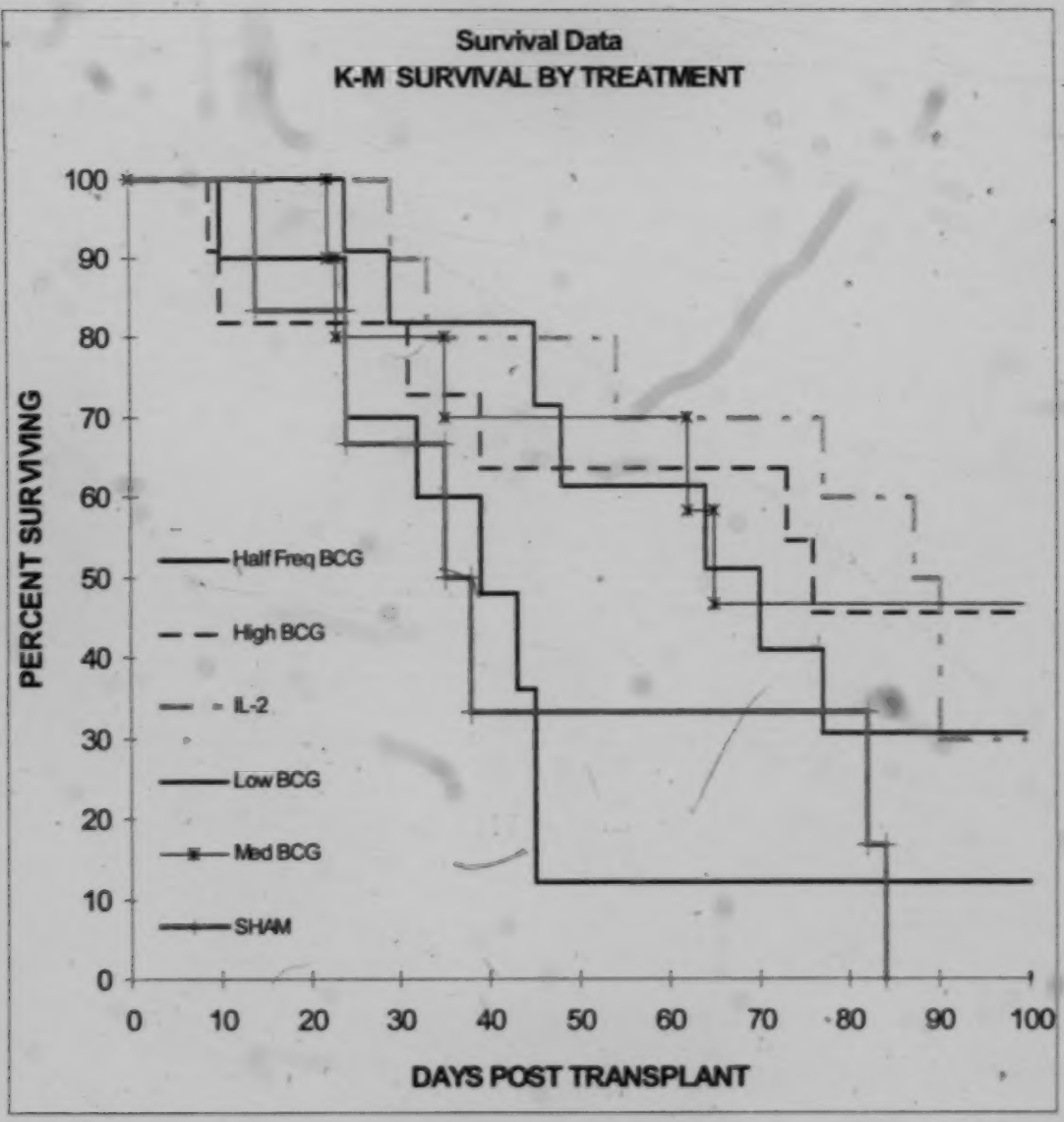


Figure 13. Kaplan-Meier (K-M) survival curves for BCG treated animals. Half-frequency BCG treatments $1x10^6$ CFU, low dose $1x10^5$ CFU, medium dose $1x10^6$CFU, high dose $1x10^7$ CFU, and IL-2 combination therapy $1x10^6$ CFU + $1x10^5$ U IL-2.

Reovirus Intravesical Therapy (Dose Response)

Animals transported to the level 3 biohazard facility tolerated cross campus travel well. Each animal acclimatized to the new environment with no noticeable alterations in behavior. All transported animals were monitored carefully by myself and the animal technicians to record changes. REO treatment groups paralleled BCG dosing, with similar PFU groups of low, medium and high administered twice per week for three weeks ($5x10^5$ – $5x10^7$). Kaplan-Meier animal survival curves were generated when the complete protocol was finished (figure 15). All REO groups showed a significant survival (students t-test) advantage relative to the Sham controls. All Sham animals were deceased by day 85 post tumor implant. Optimal REO treated animal survival was reached in the medium dose REO group with 90% of treated animals reaching LTS status. Relative risk (mortality) was lowest for REO treated groups (optimized with medium dose virus) and was substantially lower than any BCG treated group (table 5). Survival advantages in REO treated groups (figure 14) were statistically higher than all BCG groups (figure 15). All REO treated animals showed no obvious signs of distress. In some cases where hematuria was evident post implant and during early treatments, actual bleeding would subside by the fourth treatment. REO treated animals were symptom free of TCC by the third instillation of REO. In any REO treated case that was terminated prior to becoming an LTS a necropsy was performed (blinded fashion) by a university staff veterinarian.

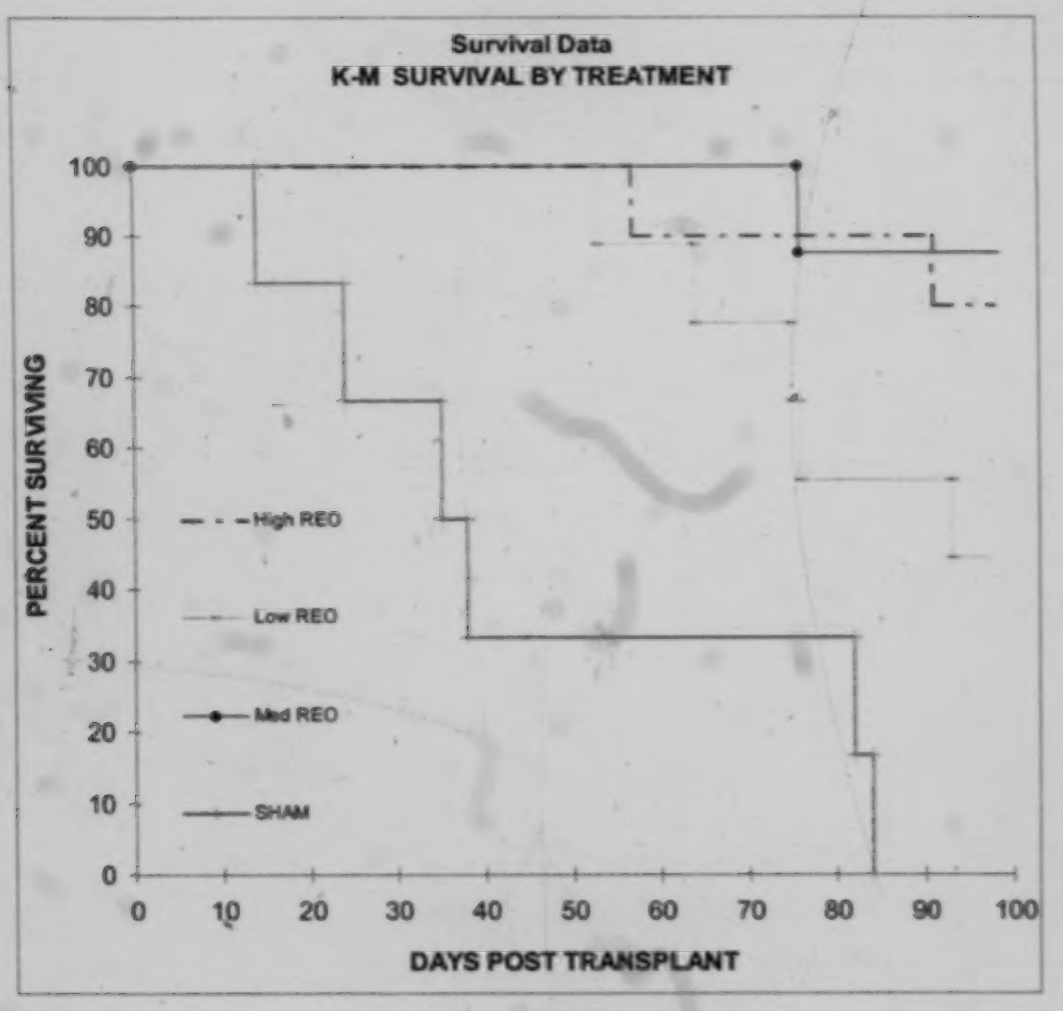


Figure 14. Kaplan-Meier (K-M) survival curves for REO treated animals. Low dose REO $1x10^5$ PFU, medium dose $1x10^6$ PFU, high dose $1x10^7$ PFU.

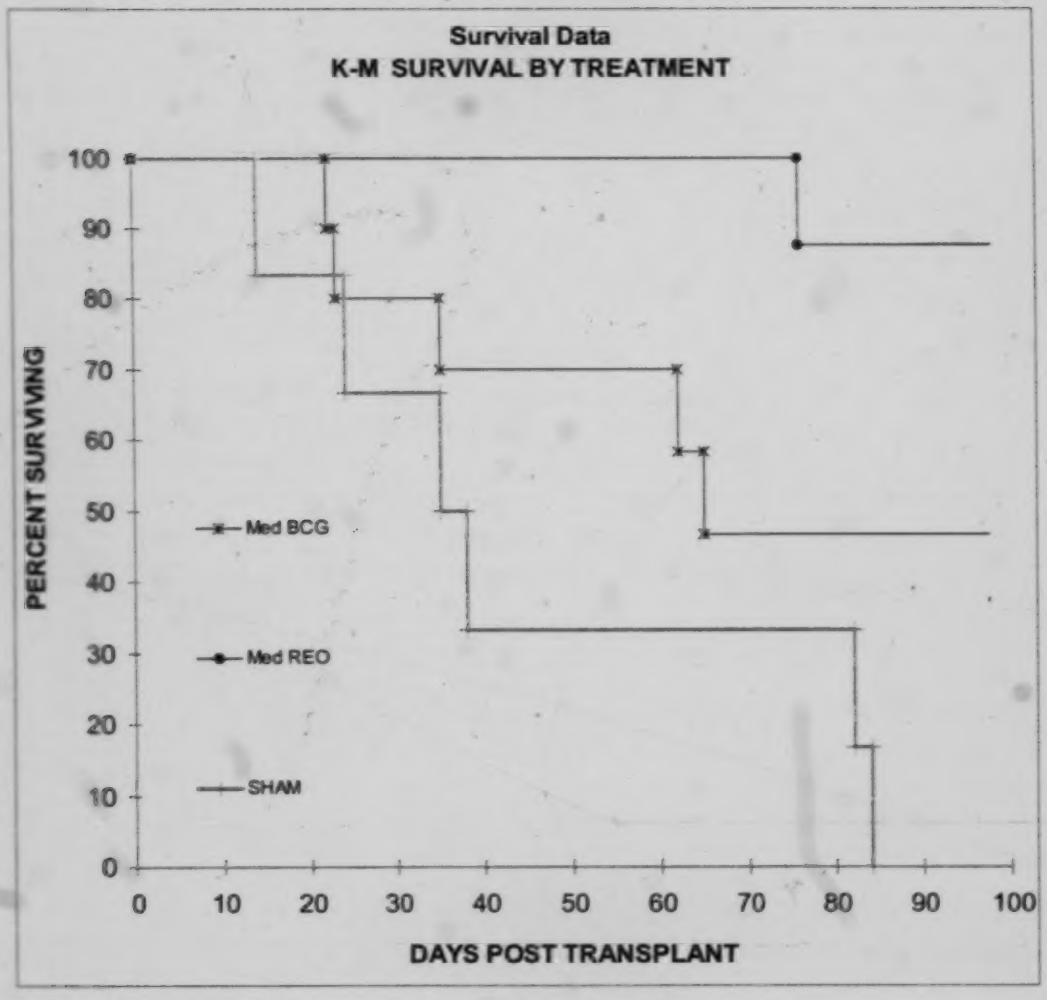


Figure 15. Kaplan-Meier (K-M) survival curves for optimal treatment regimes. Medium dose BCG and REO are included and compared with Sham controls.

Table 5. Relative risk (RR) calculated for all intravesical treatment groups relative to Sham saline controls. Comparative p values are included for each group compared with controls using a Chi squared test. All REO groups are statistically significant. Half frequency BCG instillations (1x6) were farthest from statistical significance.

COMPARISON	Chi	P-value	RR
SHAM vs Half Freq BCG	0.0635	0.8010	0.8757
SHAM vs Low BCG	2.1174	0.1456	0.4417
SHAM vs Med BCG	2.6546	0.1032	0.3866
SHAM vs High BCG	2.3262	0.1272	0.4484
SHAM vs IL-2	4.9892	0.0255	0.3091
SHAM vs Low REO	5.7176	0.0168	0.2607
SHAM vs Med REO	8.9882	0.0027	0.1961
SHAM vs High REO	13.0005	0.0003	0.1620

The medium dose REO ($5x10^6$ PFU) treated group consistently showed survival higher than controls from day 12 onward. This group was the optimal treatment dose enhancing survival in this study using with our tumor model.

Histological Analysis and Pathological Study

Bladder specimens could be easily visualized macroscopically for pathology. Both single and multiple bladder stones were commonly seen a necropsy (9%). In some cases, stones completely blocked the urethra leading to early humane termination of the animal in cases of ureter blockage (n=2 out of 9). It was not demonstrated that obstructive uropathy was caused directly by intravesical procedures or therapy. Factors graded in a blinded fashion by a clinical pathologist included degree of invasion, hemorrhage, necrosis and inflammation (table 6). Kidneys were removed from all animals at the time of necropsy. Histological results determined that chronic pyelonephritis was a rare event only occurring in one medium dose tumor positive BCG treated animal, still a LTS. Animals with bladder disease often had hydronephrosis (bilateral). There was no correlation in this study between bladder stones and hydronephrosis. Low dose BCG was most often associated with hydronephrosis (27%). No case of hydronephrosis was ever recorded in any REO treated animals. A single case of kidney infection was observed (in a medium dose BCG treated animal). No post-mortem cultures were performed to confirm and identify the causative organism for the observed infections.

A single medium dose BCG treated animal died prematurely (23 days) with a bronchiolar carcinoma. This has not been seen in this tumor model before. Because this was observed only once and never prior with this model system, the result was not pursued further. In the future, more detailed analysis of the lungs could be done to search

for possible changes or developing micro-metastasis. This lesion was never IHC analyzed to demonstrate whether or not it was a metastatic deposit.

The bladders of normal rats and sham controls were analyzed and demonstrated normal rat histology. A normal rat bladder features a thin urothelium (3-6 cell layers) and triple layered muscularis externa (figure 16). BCG treated bladders were often inflamed and showed significant levels of mononuclear cell infiltration, with or without hemorrhage (figure 16). Tumor necrosis was common in BCG and IL-2 treated bladders. Active necrosis or evidence of necrosis was seen in the majority of BCG treated animals (59%) in all doses including IL-2. Less significant signs of inflammation (never at late stage treatment) were present in REO treated animals, and necrosis was seldom present (13%), only in high dose REO treatments.

Table 6. Summary of necropsy and histology of treated animals. For cytology, - means negative, / means not done or non-diagnostic, + means positive. Not all animals were included in the histological blinded analysis. HN means hydronephrosis. Tumor indicates presence of tumor, stone means a bladder stone was found. Lymphocyte indicates there are obvious signs of neutrophil infiltration. Red blood cells (RBC) were recorded.

GROUP	N VALUES	YTOLOGY			NECROPSY			HISTOLOGY				
	TOTAL	Day 0	10	60-90	HN	Tumor	Stone	Slides	Tumor	Lymphocyte	RBC	Necrosis
1/2 BCG	10				20%	70%	0%	8	63%	38%	13%	50%
Low BCG	11	—	+++-	–+-	27%	82%	18%	N/A	N/A	N/A	N/A	N/A
Med BCG	10	–	+–	++-	20%	40%	20%	10	80%	40%	40%	60%
High BCG	11	—	+/-+	-+-/	9%	27%	36%	11	55%	18%	0%	45%
IL-2	10	-/–	-++-	-+–	20%	50%	0%	8	88%	13%	0%	88%
Low REO	9	——	/+-///	-+-///	0%	11%	0%	5	80%	20%	0%	40%
Med REO	10	——	+///+–/	-///////	0%	0%	0%	5	20%	20%	0%	0%
High REO	10	——	//+///	//////	0%	10%	10%	5	20%	40%	0%	0%
SHAM	10	—	—	—	20%	70%	0%	8	88%	50%	63%	75%

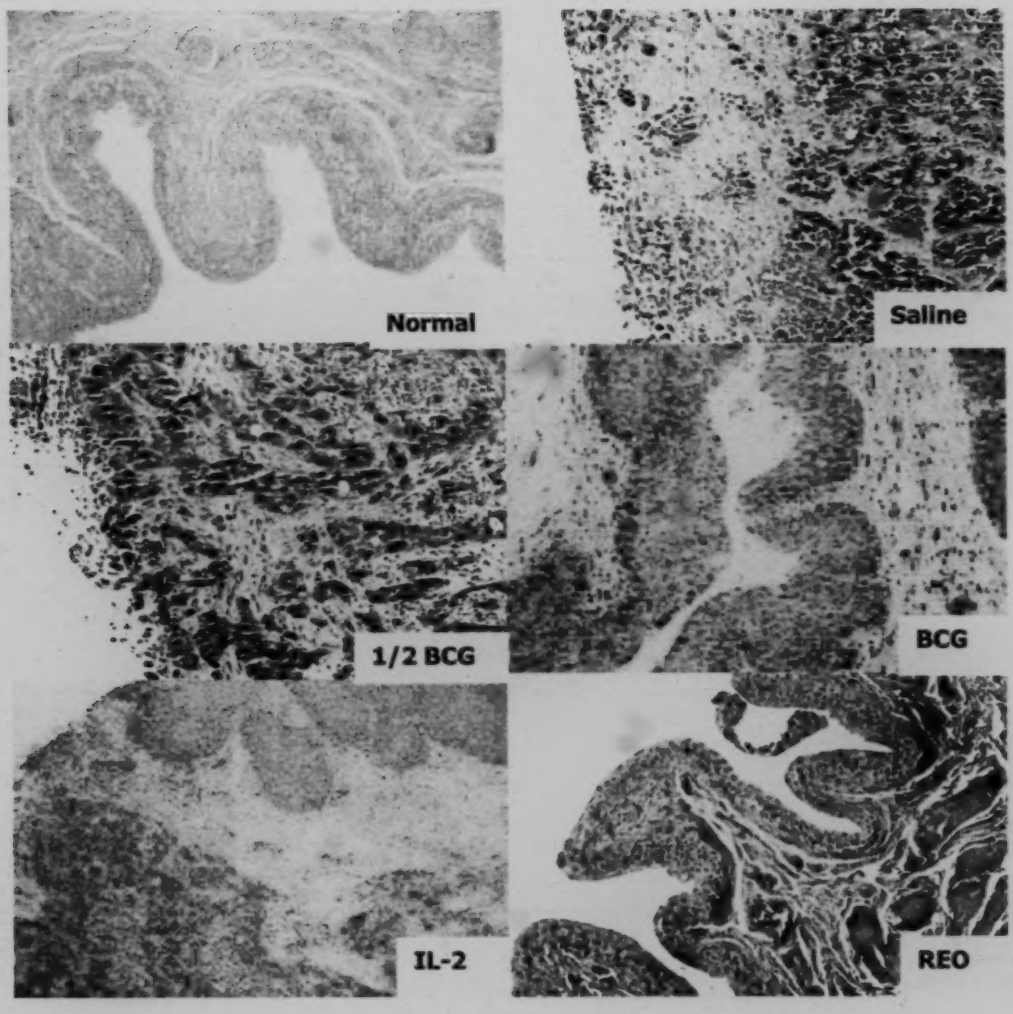


Figure 16. Histology composite of experimental animals. Treatments are indicated in each panel. Pertinent features include the nature of the untreated tumor, inflammation and tumor in BCG treatments and normal bladder architecture and lack of inflammation in REO treated animals. The Saline panel shows a high grade carcinoma. The 1/2 BCG panel shows a high grade carcinoma. BCG shows a carcinoma *in situ*. The IL-2 panel shows an invasive carcinoma. The REO panel indicates normal bladder architecture with no signs of inflammation. All images are at 20x magnification.

BCG and saline controls LTS often had remaining bladder tumors (55%). Bladder TCC transplants lead to partial replacement (multi focal) of normal urothelium with AY-27 TCC over time (10 days post-implant). This is consistent with previous characterization of the bladder tumor model for PDT studies. Modeling of the transplantable orthotopic bladder tumor model used in past research indicated three stages of growth (early, middle, and late), which corresponded to alteration of the urothelium with many cases having multiple seeding sites alongside normal urothelium. These characteristic changes in urothelium were seen in the pilot control animals (n=4) and Sham controls used throughout the experiments.

DISCUSSION

Transitional cell carcinoma, the most common form of bladder cancer remains a considerable source of mortality and morbidity worldwide [43]. Though there have been significant improvements in diagnosis and treatment, still mortality is on the rise. The major contribution to the treatment of superficial bladder cancer has been the development of intravesical therapies, namely BCG in 1976 [54]. Besides the treatment of bladder cancer, there have been many novel investigations into the genotyping of bladder tumors, which has allowed for new diagnostic and prognostic techniques. Some of these tumor markers not only allow for detection of bladder cancer but also allow for predictions to be made regarding tumor prognosis and therapeutic effectiveness. Tumor markers such as Ras and raf-1, expressed unregulated in many actively dividing cancer cells can be predictors for chemotherapy and oncolytic therapy potential (figure 1). This expression of researched tumor markers was proven to occur in our experimental cell

lines (figure 6). Because these markers (MAPK, raf-1) are expressed *in vitro* allows for the potential to test these markers and compare them with either MRI or urine cytology *in vivo* using the animal tumor model. Other markers could be tested with this system in a translational fashion before being tested in the clinic. By doing this, expensive markers would be eliminated as potential clinical markers prior to large-scale clinical studies.

Many molecular markers are available and in the process of being examined for clinical use. A large number of these markers are cell surface proteins, but also can include cytoplasmic gene products [19]. A significant step in molecular transformation of a cancer cell involves mutation of oncogenes and tumor suppressors. Proteins involved in kinase cell signaling cascades can stimulate growth and cell division/differentiation. These proteins can interact with each other by exchanging phosphate molecules (kinases and phosphatases), which can lead to activation and inactivation (figure 1). A critical kinase that is active early in the cascade web of kinases is cRaf-1 [119]. Though not a marker as such for TCC, this protein is mutated or improperly regulated in the majority of bladder cancers [105]. The first Ras kinase was discovered in bladder cancer cell lines, though often these proteins are not directly mutated in bladder cancer (<30%) the kinase cascade involving the Ras, Raf, and MAP kinase family of proteins is often (>90%) mutated or improperly regulated in bladder cancer [106]. The Ras pathway is normally involved in cell growth and differentiation. Ras kinase influences many downstream elements and is affected by many upstream elements leading to the final mitogenic effect on MAP kinase. Though this protein was isolated and quantified in this project (figure 3) it was not demonstrated to be a tumor marker per se for diagnostics. Separate studies are required to isolate tumor markers for our transplantable orthotopic bladder tumor model.

Besides usefulness as tumor markers these genes and proteins can be used for clinical predictive assays. The fact AY-27 expressed measurable amounts of different kinases (MAPK and raf-1) and was susceptible to REO oncolysis serves to add to the evidence of Ras signaling usurpation by REO for oncolysis (figure 6). Western blot analysis indicated the presence of the kinase proteins (figure 8b) and immunoprecipitation studies indicated that these kinases were functional (figure 6). It may be the functional activity of the kinase that is the prerequisite for REO oncolysis. This kinase marker (raf-1) could be used for predictive assays of response to REO in the clinic. Human TCC tested *in vitro* (MHGU3) did not respond to REO as strong as rat TCC (figure 8a). There was only one human line tested for REO response using the MTT assay in this thesis. More lines should be tested, but western analysis showed that MGHU3 cells have a low level of raf-1 expression (figure 8b). All tested cell lines should be compared to known high expressers of raf-1 kinase. Low raf-1 kinase expression and activity could be the reason for the low amount of REO oncolysis as determined by MTT. MGHU3 could be tested using much higher REO concentrations or exposure times, but the lack of high levels of kinase expression by MGHU3 could be a limiting factor for REO oncolysis. The fact MGHU3 is less susceptible to REO oncolysis, during a single exposure, may not be limiting for clinical application. REO can be administered in high doses in the bladder and for long incubation times. The fact there is any response in human TCC is promising for clinical application. Multiple exposures to REO using repeated instillations could eventually destroy a bladder tumor.

Two kinases were examined *in vitro*, raf-1 and MAPK. Previous research done by Dr. Lee et al focused on Ras kinase [120]. Many more kinases could be implicated, and a

yet to be proven final component of the Ras pathway may be the ultimate limiting factor for REO oncolysis. The scope of this preclinical project was to examine response and toxicity, the kinase assay served only as prerequisite markers of response. Being able to detect this final requirement could be useful for predicting REO responders but toxicity and clinical response are the most important factor for bringing novel therapies for phase I-III trials.

In vitro assays of the TCC line (AY-27) used in our *in vivo* tumor model did indicate that kinases (raf-1 and MAPK) implicated in carcinogenesis and cell division were expressed and active (figure 6). Relative levels were not established in comparison to normal cells due to the fact that cell lines in culture, even though not transformed, express higher levels of many kinases, including raf-1. Quiescent cells in the body do not express high levels of MAPK components (Ras, ERK1/2). Raf kinases normally stimulate cell growth and division, characteristics of cultured cells or cancerous cells *in situ*. Future research could establish primary cultures of normal bladder tissue. This was attempted as a sub-project of this research, but was unsuccessful. Multiple primary TCC cell isolations were attempted but failed in my hands. Bladder biopsies, including cancerous tissue and adjacent healthy cells were brought back to the lab from the clinic. *In vitro* culture of these samples using either monolayer overlay techniques or agitated culture at best would only produce fibroblast colonies (data not shown). Isolated TCC from humans has grown in the bladder environment in close associating to urine. There are unlimited potential urine factors that could affect bladder cancer growth. Cells isolated from human bladder cancer biopsies using a variety of primary cell isolation protocols could only at best yield scattered islets of adherent cells. This limitation may

hinder testing biopsies from patients, short term cell growth does not allow proper incubation with REO and in isolation does not allowing testing of immune dependent BCG [121].

Animal models are the ideal testing ground for bladder cancer therapies. The generation of primary TCC lines from clinical specimens is difficult and somewhat unnecessary considering how well the animal model mimics human bladder cancer. Future work could try alternate varied techniques to establish early healthy and primary TCC lines from human biopsies. Techniques beyond those attempted included varied washing and preparation mediums, different sized dishes and flasks, varied amounts of serum (10 – 30%) and variable sized biopsy specimens (with or without pretreatment with varied digestive enzymes). Tissue culture flasks pre-treated with extra cellular matrix components (fibronectin and collagen) could be used. Altering the pH of tissue culture medium might simulate a urine environment and be more favorable to cancer cell growth.

Primary lines would not have to be established to assay for REO sensitivity. Biopsies could be tested immediately after removal from the bladder using western blot analysis for various kinase proteins. Patients that have not responded to BCG in the past that are being re-tested for recurrence could undergo REO treatment prior to biopsy screening in an attempt to reduce tumor burden or remove tumor related symptoms prior to radical cystectomy. There was no evidence of REO side effects in this study (figure 16). For patients awaiting cystectomy after BCG failure, REO could be a viable treatment option to save the bladder.

The fact the cell line used in our tumor model expressed raf kinase, a participant in MAP kinase based cell signaling, indicated that oncolytic viruses may potentially infect these lines. Functional assays were used to examine kinases in TCC. The resultant activity can be interpreted to be normally active kinase, mutated overactive kinase or improperly regulated kinase expressed by the cancerous cells (figure 6). All of these scenarios ultimately lead to the same conclusion of kinase being active in cells where growth should not be normally occurring (i.e in the mature bladder). Active kinase is not always indicative of cell growth [119]. Kinases can be active in response to extracellular ligands involved in cell stress, namely interferon during viral infection [111, 122, 123].

The fact human cell lines were demonstrated to have active kinase proteins would provide evidence that translation to clinical bladder cancer could be made prior to phase I clinical experimentation. The next step to ensure productive oncolytic infection would be to carry out *in vivo* assays demonstrating viral protein translation and lytic cell processes. Because our AY-27 cell line was examined, this evidence of REO infection could be used as a basis for animal experimentation (figures 7 & 9). AY-27 TCC demonstrated a dose response to increasing levels of REO (figure 9). Human TCC cultured (MGHU3) did not show high levels of oncolysis relative to REO (figure 8). MGHU3 cells grow slower than AY-27 in culture, which may require longer incubation times with REO to allow for more replicative cycles and MGHU3 were not high expressers of raf-1 which may be required. More human TCC lines should be evaluated for REO susceptibility.

To demonstrate viral infection using REO with TCC lines in culture, immunohistochemistry was used (figure 10). The polyclonal antibody used was generated by Dr. Patrick Lee's lab in rabbits immunized with active mammalian REO serotype 3

(Dearing strain) [101]. The antibody has been characterized by Dr. Lee's lab as binding to the three outer REO capsid proteins (lambda, kappa, and sigma). In a productive virus infection these proteins are produced in large quantities before viral assembly in the cytosol. IHC allows for visualization of both cytoplasmic viral capsid proteins and extracellular viral proteins, presumably on extracellular assembled virus clusters (figure 10). It is known that assembly and aggregation of mammalian REO occurs within the cell after unregulated translation through inactivation of PKR. At this time viral particles aggregate in large intracellular inclusion bodies [104]. Using IHC with multiple levels of REO concentrations ($5x10^7$ titrated PFU/ml) and exposure times (3-7 days), cytoplasmic staining and cell destruction were observed. Clusters of stained product could be inclusion bodies forming prior to oncolysis, though photography did not clearly show this. IHC technique needs to be expanded to clearly detect REO infection. IHC did clearly show morphological processes in REO infection, which still provide valuable information regarding oncolysis. Necrosis depends on a functional immune response, which cannot be seen in culture. Morphological features of apoptosis were evident using light microscopy during IHC. Apoptosis events such as pyknosis occurred as early as day 5 (figure 10). Cellular destruction could be caused by either cell lysis or induced apoptosis. Apoptosis could be the mechanism by which REO destroys cancer cells *in vivo* also. Histology did not reveal any signs of bladder scarring in REO treated animals. In all REO animals, only 2 cases of necrosis were seen. These animals also had bladder tumors, suggesting that REO may still be acting in these animals producing an anti-tumor response (not tested). REO may selectively kill bladder cancer cells via apoptosis with

limited necrosis. The level of necrosis would have to be extremely low in order to allow the bladder to return to the same level of function it had prior to treatment.

In normal cells, PKR is active and once autophosphorylated will inhibit viral protein translation. PKR inactivation can lead to apoptosis [111]. In cases of REO infection it is known that post-assembly (cellular inclusions) and early virus release can lead to apoptosis [109]. This has significant implications for potential use of REO as a human therapy for any cancer. The key feature of cell death from apoptosis is the lack of an immune inflammatory response. The fact that there is no inflammation with apoptosis makes therapies involving activation of apoptosis (as a mechanism for tumor destruction) very attractive. Histologically, apoptosis seemed to be the mechanism by which REO selectively killed TCC in our animal model. Such therapies would be devoid of inflammatory side effects seen with many anti-cancer therapies, including intravesical BCG.

Early IHC staining (prior to day 2) detected in the AY-27 cells could be the result of internalized REO pro-particles, as endosomal entry into mammalian cells is receptor dependent [124]. Research done early in REO infection studies indicated that REO was able to bind and internalize through the EGFR pathway [103]. It has since been elucidated that REO is able to bind a wide variety of glycosylated cell surface proteins common to a variety of mammalian cells. Bladder tumors are known to express EGFR [125]. Infection of EGFR positive cells would only ensure REO attachment and internalization. An infection in cells with an active PKR would not be productive; the REO genes would not be translated and packaged into active virus progeny required for lytic or apoptotic infection (figure 3).

Co-culture studies illustrated another advantage of REO therapy. Delivery of anti-cancer agents of any form is a barrier to an effective treatment. REO can actively infect cells thereby propagating therapy to continue on after the initial administration. As REO infects a mammalian cell with an abnormal PKR system, more progeny virus are produced that can then infect neighboring cells. This propagation of infection is unique to viral therapies. This effect can be visualized in the MCS co-culture study. REO destroys the TCC that normally surrounds the healthy fibroblasts. REO in the bladder would have topical access to urothelium and TCC patches. Large doses of REO could be used and the GAG layer of the bladder may actually serve to hold REO in place to allow attachment and internalization. The bladder offers a unique location to instill chemotherapies and REO exploits all possible advantages.

The common diagnostic factor remains patient presentation with symptoms of hematuria [35]. Cystoscopy is the mainstay of obtaining a biopsy for pathological grading and for confirming a TCC diagnosis. Even after treatment, cystoscopy must be routinely performed because many TCC tumors will recur. It is the very nature of recurrence in bladder cancer cases that require the development of novel treatments that can be repeated when tumors recur. Cystoscopy is an invasive procedure; treatments that could reduce its use in the clinical would be invaluable. Diagnostic cystoscopy can traumatize the urothelium or the urethra. This can potentially complicate subsequent adjuvant immunotherapies using BCG or other immune stimulants (IL-2). Trauma either prior to or during BCG instillation can increase systemic side effects. A traumatized urothelium can provide a more patent route to the circulation for the mycobacterium or other topical agents. Complications associated with BCG are more often linked with a traumatized

catheterization at the time of instillation. Ultimately this can lead to a systemic infection known as BCGosis or BCGitis. In our experiments, there was no obvious case of systemic BCGosis, though specific tests for systemic infection were not carried out. In all BCG treatment groups combined only 10 cases of hydronephrosis occurred (table 6). This finding could be due to obstruction secondary to tumor or bladder stones rather than infection. Routine blood work on experimental animals is not feasible or was it justified in these experiments. The goal was to test for the potential survival advantages conferred in the model for either BCG or REO therapy. No cultures were done in this research project but could be incorporated into future BCG studies using this tumor model. Future experimentation using our model system and BCG instillation could include specific cultured and molecular assays for BCG infection. In such an experiment BCG side effects could be more closely studied or monitored. Classic signs of BCG infection include granuloma formation, commonly in the inguinal lymph nodes. At necropsy there was no evidence of granuloma formations (table 6). Swollen inguinal nodes were not seen in any of the treated animals or sham controls.

BCG treated animals presented with symptoms that indicated animal stress early during the 6 week treatment period (piloerection). The intravesical treatments required that each animal was catheterized multiple times, which could have lead to micro-trauma in the urethra and bladder. There is no way to evaluate this micro trauma without dissecting the bladder and examining the histology. Although not readily visibly or detectable, these micro abrasions could lead to BCG related complications. Animals were visibly stressed, though otherwise tolerated the BCG therapy (table 6). Animals still feed and drank throughout treatment. Only in the endpoint prior to euthanization did animals

stop exhibiting normal homeostasis type activities. The signs of stress did not diminish between treatments or after the final treatment. Few BCG treated animals remained alive for more than 1 month after the last treatment.

Animal weights were recorded prior to tumor implant and were monitored at least once per week throughout treatment (data not shown). There were no statistical differences in animal weight between groups or within groups. This was done to aid in quantifying signs of bladder cancer and overall general health. In the past, animal body weight has been a poor predictor of tumor development and usually did not correlate with tumor positive animals. This indicated that tumor growth was relatively constant, i.e. no large tumor masses rapidly grew to contribute significantly to body mass. All animals gained some degree of weight; therefore there was no precipitous weight gain or loss. Weight is also a general indicator of animal health. Previous work with our bladder tumor model only demonstrated weight loss to occur in very late stage cancer. At these late time points in the model, there is already a palpable tumor mass in the lower abdomen of the animal. Also at this point the animal has already lost significant body mass, and appears to have a very wasted body (low body fat for age). Future studies could monitor body weight with continuous regular MRI scans and body fat analysis (caliper studies). This type of research could help elucidate more features of tumor development in this model, though including MRI studies, especially frequent monitoring would present more complications and cost. The MRI available for research use is also used for clinical investigations, animal monitoring cannot be done at any regular interval. The information gained from such a study would not alter information gleaned from this intravesical therapy experiment.

Urine cytology, in combination with urinalysis is the gold standard of diagnosing bladder cancer. To reduce on animal housing costs a single cage held two animals. This meant hematuria could only be recorded on a per cage basis. Individual animals could not be scored as being hematuria positive or negative. In some cases prior to treatment, evidence of hematuria could be obviously seen at time of catheterization. This was not necessarily indicative of actual bladder bleeding arising from bladder tumor. This could be the result of previous catheterizations or urethra trauma or scarring. All animals were treated regardless of urethra condition. Often, difficulty in catheterizing an animal was a sign that a late stage tumor was developing. This was usually followed by early endpoint or humane euthanasia.

In the majority of cases, urine was present and drained once the catheter was inserted into the bladder and cells were detectable (figure 12). When this was not the case, the bladder was washed with 0.9% normal saline solution. Roughly 0.5 cc of saline is required for an average sized rat bladder wash. This volume corresponds with urodynamic studies previously done in Dr. Moore's lab [115]. Often when blood was not visible in the collected urine or saline wash, a standard urine hematuria dipstick could detect it, or red blood cells (RBC) would be clearly demonstrated in centrifuged samples used for urine cytology screening. In this study, it was very rare when a bladder cancer positive animal presented without hematuria (none).

MRI imaging is a costly and lengthy involved procedure. Two animals can be imaged at the same time using a standard clinical knee bracket. Many times imaging had to be done on a clinical schedule, meaning some animals were not imaged at an exact time point, i.e. not all animals underwent MRI at day ten. Another issue with

experimental MRI for monitoring animals is that each image requires the animals be anesthetized and catheterized. For BCG studies, repeat catheterizations can lead to urological trauma, which can increase BCG related side effects and toxicity. The length of time it takes to MRI an animal requires complete general anesthesia, which puts a research rat at surgical plane for approximately one hour at standard dose. Collecting urine is also done under anesthesia, but can be performed just prior to an intravesical treatment, also cytology can be drawn using short acting inhalant anesthesia. Though more expensive, inhalant anesthesia (isoflurane) is less severe than parenteral anesthesia and only affects the animal for a few minutes, while still allowing surgical plane anesthesia levels.

In this study, urine cytology was not a reliable method of detecting bladder cancer (figure 12). It is important that research models mimic the clinical disease they are used to study. Urine cytology, prior to cystoscopy, is a gold standard of diagnosing bladder cancer in humans. As with clinical specimens, the cytology reports from our animal model showed the same diagnostic features. Changes in these diagnostic features could be another potential research arm using this animal model. Many experimental intravesical therapies are monitored using urine markers and cytology [35, 113].

In situ (cis) tumors are known to shed cells detectable by urine cytology more often than T1 or Ta. Even though Tis tumors shed more cells in the urine, this is the tumor type most likely to be missed using cystoscopy [19]. Reliable routine collection of urine is a problem in experimental animal models. For difficult catheterizations, urine (up to 1 ml) is lost through the catheter while secure access is established. In these instances, the animal usually has tumor complications (partially obstructed urethra). Surgical plane

anesthesia results in loss of sphincter tone and urine is also lost manipulating the animal to the surgical table. Urine is also lost just in routine animal handling prior to anesthesia (i.e. during weighing the animal). In these cases, bladder washes may not provide the proper cell density needed for cytological screening. Most tumor cells in the urine are lost in the initial voided urine volume. A complete cytology study using our animal model could provide the proper evaluation of cytology as an experimental screener. Animals could be implanted with TCC and then screened for TCC that could be later confirmed by necropsy. This would leave the evaluation of urine cytology without the added variable of an intravesical study done on the same animals. Intravesical therapy could alter the results or urine cytology; therefore a separate study into the feasibility of cytology in this animal model is warranted. Such a study may not be an ethical use of animals, in our study urine cytology served the purpose of adding evidence of tumor presence, already building on the historical statistical 90% tumor take in our transplantable TCC model system [115]. The pilot implant series of 4 animals did prove the experimental technique was sound and the tumor model was effective.

BCG was not well tolerated by treated animals. Early signs of stress were evident as early 24 hours after BCG instillation. The early sign of stress included piloerection and animal hunching. Normally with this model all animals recover nicely after implant within hours with no signs of stress. Though there was a survival advantage using BCG relative to Sham controls, side effects were seen that were not observed in REO treated groups (figure 13 & 14). These side effects mainly involved the persistence of cancerous cells in BCG treated animal, even in many LTS (figure 15). These animals showed overt signs of stress compared with REO treated animals. This could be due to the difference in

anti-tumor mechanisms between BCG and REO. BCG is a known non-specific immune stimulator. Such a non-specific activation of immune cells (LAK) is accompanied by an inflammation response. It is this inflammation that underlies side effects seen in BCG treatments [59]. The small animal tumor model used in this research could be used to study the mechanism of action of BCG in hopes to find ways to further reduce side effects. Intravesical therapy research for bladder cancer aims to either find novel therapies such as REO or to reduce side effects of BCG.

The most significant result of this research project was the tumor cure seen in REO treated animals. In the optimal REO group, 9/10 of the treated animals were LTS (figure 15). Statistically based on historical evidence, at worst only one of these animals would be expected to have a failed tumor implant. The reversal of disease in this group was evident. Animals tolerated the REO instillations very well and animal behavior was not affected adversely as was the case in BCG treated animals. Many of the REO treatment grouped animals exhibited signs of distress around day 7 post-implant and just prior to transport to HSLAS (subjective observations). During early treatments (around day 10), all animals still exhibited signs of distress. Even hematuria was present during the initial two treatments of REO treated animals. By the third treatment, these signs would be absent. The lack of mid-treatment hematuria was not seen in BCG treated animals. This was the most striking differential observation made between REO and BCG treated groups. Animals originally appearing stressed would then be recorded by daily monitoring as being perfectly healthy by the third REO instillation. The difference between early animal stresses in all animals was that BCG symptoms never subsided. Because animal sentinels were present in both health care facilities, differences due to

housing were not a factor. No signs of systemic viral infection were ever present in any of the animals. Also, both animal care facilities (CCI clean conventional, and HSLAS Biohazard Level 3) were kept under strict CACC guidelines that are uniform and regulated.

Histological results confirmed observations during and after treatment. Inflammation was evident, as was residual tumor in BCG treated groups (figure 16). IL-2 made no histological difference when compared with BCG treated groups. There was evidence that IL-2 boosted the inflammatory response seen with the complimentary BCG group (low). The majority (7/8) of IL-2 treated bladders showed histological evidence of severe necrosis (table 6). IL-2 may be increasing the immune response to BCG induced inflammation to dangerous levels. This might lead to increased side effects, as most BCG side effects are caused by inflammation. There was only a single BCG/IL-2 combination group. Future experiments could provide multiple dosing of combination therapy using various other immune stimulators. Cytokines administered with BCG could also involve multiple routes of entry (intraperitoneal or intravenous). Intravesical administration in conjunction with BCG or engineered BCG producing IL-2 would be an elegant therapy. Therefore, intravesical administration was used in this experiment. Extra therapeutic adjuncts could complicate BCG therapy but could be evaluated in future studies. An important characteristic of any novel cancer treatment is simplicity. REO can be instilled in the same manner as BCG, which is already in wide spread use in the clinics. Future studies could monitor the potential to use REO as a second line of therapy after repeated BCG administrations have failed. REO could also be used as a final potential alternative to radical cystectomy.

There exists potential to use isolates of REO (capsid proteins) as an alternative to complete (whole) REO particles. This may reduce any side effects that could arise in phase I clinical REO trails. Though no side effects were seen in this study, attributable to REO treatments, human trials may yield different outcomes. The problem with isolating individual REO proteins is that each protein in single use may not induce an anti-tumor effect and could still cause immunological side effects, since capsid proteins are themselves epitopes to the immune system. The production costs of complete REO are minimal; isolating distinct particles (capsid proteins) will increase these costs unnecessarily. Currently REO is easy to manufacture in the large amounts required for human trials. However, at present, most GLP facilities are only capable of producing small batches in accordance to the guidelines set out by the Health Protection Branch of the Canadian Government.

In summary, long term side effect free survival was a more prominent outcome in all REO treated animals (figures 13 & 14). This was shown statistically with survival curves and using relative risk analysis (table 5 & figure 15). This level of survival has only been seen in PDT treated animals. The use of current therapies (BCG) in an experimental model provides a measure and allows novel therapies (REO) to be compared. BCG offered no real long-term survival but appeared to delay tumor progression. BCG should be examined further in this experimental model using different dosing and treatment schedules to study side effects in more detail. Acute REO side effects were nonexistent relative to BCG. Long term REO side effects could be examined in animals that are kept for extended periods after treatment. A repeat of this project with prolonged survival time recordings could also study recurrence and carcinogenesis. This

would involve periodic monitoring of the animals for tumor development, which could be done using MRI. In conclusion REO as an intravesical therapy for TCC was well tolerated and superior to BCG in this preclinical study and should therefore be further evaluated in phase I/II clinical studies.

References

1. Oosterlinck, W., *The management of superficial bladder cancer.* BJU Int, 2001. **87**(2): p. 135-40.
2. Murphy, W.M., *The term 'superficial bladder cancer' should be abandoned.* Eur Urol, 2000. **38**(5): p. 597-9.
3. Spruck, C.H., 3rd, et al., *Two molecular pathways to transitional cell carcinoma of the bladder.* Cancer Res, 1994. **54**(3): p. 784-8.
4. Hurle, R., et al., *Upper urinary tract tumors developing after treatment of superficial bladder cancer: 7-year follow-up of 591 consecutive patients.* Urology, 1999. **53**(6): p. 1144-8.
5. Oyasu, R., *Epithelial tumours of the lower urinary tract in humans and rodents.* Food Chem Toxicol, 1995. **33**(9): p. 747-55.
6. Statistics, C., in *National Cancer Institute of Canada.* 1996. p. 7-47.
7. Millan-Rodriguez, F., et al., *Primary superficial bladder cancer risk groups according to progression, mortality and recurrence.* J Urol, 2000. **164**(3 Pt 1): p. 680-4.
8. Altay, B., et al., *The best management of superficial bladder tumours: comparing TUR alone versus TUR combined with intravesical chemotherapy modalities?* Int Urol Nephrol, 2000. **32**(1): p. 53-8.
9. Saint, F., et al., *Tolerability of bacille Calmette-Guerin maintenance therapy for superficial bladder cancer.* Urology, 2001. **57**(5): p. 883-8.
10. Hassen, W. and M.J. Droller, *Current concepts in assessment and treatment of bladder cancer.* Curr Opin Urol, 2000. **10**(4): p. 291-9.
11. Schulte, P.A., *The role of genetic factors in bladder cancer.* Cancer Detect Prev, 1988. **11**(3-6): p. 379-88.
12. Kiemeney, L.A. and M. Schoenberg, *Familial transitional cell carcinoma.* J Urol, 1996. **156**(3): p. 867-72.
13. Kramer, A.A., et al., *Familial aggregation of bladder cancer stratified by smoking status.* Epidemiology, 1991. **2**(2): p. 145-8.
14. Lee, R. and M.J. Droller, *The natural history of bladder cancer. Implications for therapy.* Urol Clin North Am, 2000. **27**(1): p. 1-13, vii.
15. el-Mawla, N.G., M.N. el-Bolkainy, and H.M. Khaled, *Bladder cancer in Africa: update.* Semin Oncol, 2001. **28**(2): p. 174-8.
16. Ernster, V.L., *Trends in smoking, cancer risk, and cigarette promotion. Current priorities for reducing tobacco exposure.* Cancer, 1988. **62**(8 Suppl): p. 1702-12.
17. Fujiyama, C., et al., *Human bladder cancer invasion model using rat bladder in vitro and its use to test mechanisms and therapeutic inhibitors of invasion.* Br J Cancer, 2001. **84**(4): p. 558-64.
18. Malmstrom, P.U., *Management of superficial bladder cancer: what is new?* Curr Opin Urol, 2000. **10**(5): p. 447-51.
19. Cordon-Cardo, C., R.J. Cote, and G. Sauter, *Genetic and molecular markers of urothelial premalignancy and malignancy.* Scand J Urol Nephrol Suppl, 2000. **163**(205): p. 82-93.
20. Oshinsky, G.S., et al., *A model of bladder tumor xenografts in the nude rat.* J Urol, 1995. **154**(5): p. 1925-9.

21. Crook, T.J., et al., *A model of superficial bladder cancer using fluorescent tumour cells in an organ-culture system.* BJU Int, 2000. **86**(7): p. 886-93.
22. Sidransky, D., et al., *Clonal origin bladder cancer.* N Engl J Med, 1992. **326**(11): p. 737-40.
23. Dalbagni, G., et al., *Molecular genetic alterations of chromosome 17 and p53 nuclear overexpression in human bladder cancer.* Diagn Mol Pathol, 1993. **2**(1): p. 4-13.
24. Llopis, J., et al., *p53 expression predicts progression and poor survival in T1 bladder tumours.* Eur Urol, 2000. **37**(6): p. 644-53.
25. Baithun, S.I., et al., *Molecular and kinetic features of transitional cell carcinomas of the bladder: biological and clinical implications.* Virchows Arch, 2001. **438**(3): p. 289-97.
26. Baselli, E.C. and R.E. Greenberg, *Intravesical therapy for superficial bladder cancer.* Oncology (Huntingt), 2000. **14**(5): p. 719-29; discussion 729-31, 734, 737.
27. Raghavan, D. and R. Huben, *Management of bladder cancer.* Curr Probl Cancer, 1995. **19**(1): p. 1-64.
28. Pages, F., et al., *p53 status does not predict initial clinical response to bacillus Calmette-Guerin intravesical therapy in T1 bladder tumors.* J Urol, 1998. **159**(3): p. 1079-84.
29. Shariat, S.F., et al., *Prognostic value of P53 nuclear accumulation and histopathologic features in T1 transitional cell carcinoma of the urinary bladder.* Urology, 2000. **56**(5): p. 735-40.
30. Simon, R., et al., *High-throughput tissue microarray analysis of 3p25 (RAF1) and 8p12 (FGFR1) copy number alterations in urinary bladder cancer.* Cancer Res, 2001. **61**(11): p. 4514-9.
31. Krause, F.S., et al., *Molecular genetic methods in the diagnosis of invasive bladder cancer.* Anticancer Res, 2000. **20**(6D): p. 5015-21.
32. Brake, M., et al., *Recurrence and progression of stage T1, grade 3 transitional cell carcinoma of the bladder following intravesical immunotherapy with bacillus Calmette-Guerin.* J Urol, 2000. **163**(6): p. 1697-701.
33. Herr, H.W., *Natural history of superficial bladder tumors: 10- to 20-year follow-up of treated patients.* World J Urol, 1997. **15**(2): p. 84-8.
34. Volante, M., et al., *Progression from superficial to invasive carcinoma of the bladder: Genetic evidence of either clonal heterogeneous events.* Hum Pathol, 2001. **32**(5): p. 468-74.
35. Koenig, F., et al., *Urinary markers of malignancy.* Clin Chim Acta, 2000. **297**(1-2): p. 191-205.
36. Konety, B.R. and R.H. Getzenberg, *Urine based markers of urological malignancy.* J Urol, 2001. **165**(2): p. 600-11.
37. Mungan, N.A., et al., *Urinary bladder cancer test: a new urinary tumor marker in the follow-up of superficial bladder cancer.* Urology, 2000. **56**(5): p. 787-92.
38. Cairns, P., M.E. Shaw, and M.A. Knowles, *Initiation of bladder cancer may involve deletion of a tumour- suppressor gene on chromosome 9.* Oncogene, 1993. **8**(4): p. 1083-5.

39. Nakopoulou, L., et al., *MMP-3 mRNA and MMP-3 and MMP-1 proteins in bladder cancer: a comparison with clinicopathologic features and survival.* Appl Immunohistochem Molecul Morphol, 2001. **9**(2): p. 130-7.
40. Oge, O., et al., *Comparison of BTA stat and NMP22 tests in the detection of bladder cancer.* Scand J Urol Nephrol, 2000. **34**(6): p. 349-51.
41. Zlotta, A.R. and C.C. Schulman, *Biological response modifiers for the treatment of superficial bladder tumors.* Eur Urol, 2000. **37**(Suppl 3): p. 10-5.
42. Soloway, M.S., *Intravesical therapy for bladder cancer.* Urol Clin North Am, 1988. **15**(4): p. 661-9.
43. Kurth, K.H., et al., *Treatment of superficial bladder tumors: achievements and needs. The EORTC Genitourinary Group.* Eur Urol, 2000. **37**(Suppl 3): p. 1-9.
44. Morales, A., J.L. Chin, and E.W. Ramsey, *Mycobacterial cell wall extract for treatment of carcinoma in situ of the bladder.* J Urol, 2001. **166**(5): p. 1633-7; discussion 1637-8.
45. Zlotta, A.R., et al., *What are the immunologically active components of bacille Calmette-Guerin in therapy of superficial bladder cancer?* Int J Cancer, 2000. **87**(6): p. 844-52.
46. Nseyo, U.O., et al., *Photodynamic therapy (PDT) in the treatment of patients with resistant superficial bladder cancer: a long-term experience.* J Clin Laser Med Surg, 1998. **16**(1): p. 61-8.
47. Tseng, M.T., et al., *The influence of photodynamic therapy on the ultrastructure of the normal rat bladder.* J Photochem Photobiol B, 1991. **9**(3-4): p. 295-305.
48. Sharman, W.M., C.M. Allen, and J.E. van Lier, *Photodynamic therapeutics: basic principles and clinical applications.* Drug Discov Today, 1999. **4**(11): p. 507-517.
49. Korbelik, M. and J. Hung, *Cellular delivery and retention of Photofrin II: the effects of interaction with human plasma proteins.* Photochem Photobiol, 1991. **53**(4): p. 501-10.
50. Xiao, Z., et al., *Biodistribution of Photofrin II and 5-aminolevulinic acid-induced protoporphyrin IX in normal rat bladder and bladder tumor models: implications for photodynamic therapy.* Photochem Photobiol, 1998. **67**(5): p. 573-83.
51. Bohle, A., *BCG's mechanism of action - increasing our understanding. For the EBIN Group.* Eur Urol, 2000. **37**(Suppl 1(6)): p. 1-8.
52. Mathe, G., et al., *Active immunotherapy for acute lymphoblastic leukaemia.* Lancet, 1969. **1**(7597): p. 697-9.
53. Matthews, N. and J.F. Watkins, *Tumour-necrosis factor from the rabbit. I. Mode of action, specificity and physicochemical properties.* Br J Cancer, 1978. **38**(2): p. 302-9.
54. Morales, A., D. Eidinger, and A.W. Bruce, *Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors.* J Urol, 1976. **116**(2): p. 180-3.
55. Lamm, D.L., et al., *Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer.* J Urol, 1992. **147**(3): p. 596-600.
56. Ratliff, T.L., L.R. Kavoussi, and W.J. Catalona, *Role of fibronectin in intravesical BCG therapy for superficial bladder cancer.* J Urol, 1988. **139**(2): p. 410-4.

57. Kuroda, K., et al., *Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells.* J Clin Invest, 1993. **91**(1): p. 69-76.
58. Morcos, E., et al., *Bacillus Calmette-Guerin induces long-term local formation of nitric oxide in the bladder via the induction of nitric oxide synthase activity in urothelial cells.* J Urol, 2001. **165**(2): p. 678-82.
59. Bohle, A., et al., *Reduction of side effects of intravesical therapy with bacille Calmette-Guerin by pentoxifylline?--an in vitro approach.* Clin Infect Dis, 2000. **31** Suppl **3**(3): p. S101-5.
60. Schmitz-Drager, B.J. and M. Muller, *Intravesical treatment of bladder cancer: current problems and needs.* Urol Int, 1998. **61**(4): p. 199-205.
61. Leibovici, D., et al., *Elevated prostate specific antigen serum levels after intravesical instillation of bacillus Calmette-Guerin.* J Urol, 2000. **164**(5): p. 1546-9.
62. Ratliff, T.L., D. Gillen, and W.J. Catalona, *Requirement of a thymus dependent immune response for BCG-mediated antitumor activity.* J Urol, 1987. **137**(1): p. 155-8.
63. Zlotta, A.R., et al., *Superficial bladder tumors and increased reactivity against mycobacterial antigens before bacillus Calmette-Guerin therapy.* J Urol, 1998. **159**(6): p. 1885-91.
64. Bassi, P., et al., *Modified induction course: a solution to side-effects?* Eur Urol, 2000. **37**(Suppl 1(7)): p. 31-2.
65. Malkowicz, S.B., *Intravestical therapy for supervical bladder cancer.* Semin Urol Oncol, 2000. **18**(4): p. 280-8.
66. Bassi, P., *BCG (Bacillus of Calmette Guerin) therapy of high-risk superficial bladder cancer.* Surg Oncol, 2002. **11**(1-2): p. 77-83.
67. Jansson, O.T., et al., *The role of nitric oxide in bacillus Calmette-Guerin mediated anti-tumour effects in human bladder cancer.* Br J Cancer, 1998. **78**(5): p. 588-92.
68. Tan, L., G. Testa, and T. Yung, *Diffuse alveolar damage in BCGosis: a rare complication of intravesical bacillus Calmette-Guerin therapy for transitional cell carcinoma.* Pathology, 1999. **31**(1): p. 55-6.
69. Numao, N., S. Goto, and S. Suzuki, *[A case of renal tuberculosis following bacillus Calmette-Guerin instillation therapy for bladder cancer].* Hinyokika Kiyo, 2000. **46**(2): p. 109-11.
70. Ozbakkaloglu, B., et al., *Granulomatous hepatitis following intravesical bacillus Calmette-Guerin therapy.* Int Urol Nephrol, 1999. **31**(1): p. 49-53.
71. Frey, H.M., *Septic shock in a 31-year-old male with a superficial bladder tumor.* Int J Infect Dis, 1998. **2**(4): p. 226-9.
72. Collado, A., et al., *Early complications of endoscopic treatment for superficial bladder tumors.* J Urol, 2000. **164**(5): p. 1529-32.
73. Al Khalifa, M., et al., *The effect of isoniazid on BCG-induced toxicity in patients with superficial bladder cancer.* Eur Urol, 2000. **37**(Suppl 1): p. 26-30.
74. Mack, D. and J. Frick, *Low-dose bacille Calmette-Guerin (BCG) therapy in superficial high-risk bladder cancer: a phase II study with the BCG strain Connaught Canada.* Br J Urol, 1995. **75**(2): p. 185-7.

75. van Gils-Gielen, R.J., et al., *Risk factors in carcinoma in situ of the urinary bladder. Dutch South East Cooperative Urological Group.* Urology, 1995. **45**(4): p. 581-6.
76. Bohle, A. and C. Durek, *Recent perspectives in topical therapy in superficial bladder cancer.* Curr Opin Urol, 1999. **9**(5): p. 407-11.
77. Pagano, F., et al., *Improving the efficacy of BCG immunotherapy by dose reduction.* Eur Urol, 1995. **27**(Suppl 1(4)): p. 19-22.
78. Vegt, P.D., F.M. Debruyne, and A.P. van der Meijden, *Bacillus Calmette-Guerin in superficial bladder cancer: consensus and controversies.* Eur Urol, 1995. **27**(2): p. 89-95.
79. Zlotta, A.R., et al., *What is the optimal regimen for BCG intravesical therapy? Are six weekly instillations necessary?* Eur Urol, 2000. **37**(4): p. 470-7.
80. Andres, E., et al., *[Sepsis syndrome and bone marrow granulomatosis after intravesical instillation of BCG].* Presse Med, 1999. **28**(32): p. 1753-4.
81. Akaza, H., *BCG treatment of existing Ta, T1 tumours or carcinoma in situ of the bladder.* Eur Urol, 1995. **27**(Suppl 1(1)): p. 9-12.
82. Brake, M., et al., *Long-term results of intravesical bacillus Calmette-Guerin therapy for stage T1 superficial bladder cancer.* Urology, 2000. **55**(5): p. 673-8.
83. Lamm, D.L., et al., *Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study.* J Urol, 2000. **163**(4): p. 1124-9.
84. Shelley, M.D., et al., *Intravesical Bacillus Calmette-Guerin in Ta and T1 Bladder Cancer (Cochrane Review).* Cochrane Database Syst Rev, 2000. **101**(4).
85. Pow-Sang, J.M. and J.D. Seigne, *Contemporary management of superficial bladder cancer.* Cancer Control, 2000. **7**(4): p. 335-9.
86. Malmstrom, P., *Improved patient outcomes with BCG immunotherapy vs. chemotherapy - Swedish and worldwide experience.* Eur Urol, 2000. **37**(Suppl 1(6)): p. 16-20.
87. Shin, J.S., et al., *Induction of tumour necrosis factor-alpha (TNF-alpha) mRNA in bladders and spleens of mice after intravesical administration of bacillus Calmette-Guerin.* Clin Exp Immunol, 1995. **100**(1): p. 26-31.
88. Ratliff, T.L., et al., *T-cell subsets required for intravesical BCG immunotherapy for bladder cancer.* J Urol, 1993. **150**(3): p. 1018-23.
89. Jurincic-Winkler, C.D., et al., *Keyhole limpet hemocyanin for carcinoma in situ of the bladder: a long-term follow-up study.* Eur Urol, 2000. **37**(Suppl 3): p. 45-9.
90. Mulvihill, S., et al., *Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial.* Gene Ther, 2001. **8**(4): p. 308-15.
91. Chen, Y., et al., *Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy.* Hum Gene Ther, 2000. **11**(11): p. 1553-67.
92. Galanis, E., R. Vile, and S.J. Russell, *Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors.* Crit Rev Oncol Hematol, 2001. **38**(3): p. 177-92.

93. Coukos, G., et al., *Oncolytic herpes simplex virus-1 lacking ICP34.5 induces p53-independent death and is efficacious against chemotherapy-resistant ovarian cancer.* Clin Cancer Res, 2000. **6**(8): p. 3342-53.
94. Pawlik, T.M., et al., *Oncolysis of diffuse hepatocellular carcinoma by intravascular administration of a replication-competent, genetically engineered herpesvirus.* Cancer Res, 2000. **60**(11): p. 2790-5.
95. Yoon, S.S., et al., *An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma.* Faseb J, 2000. **14**(2): p. 301-11.
96. Cozzi, P.J., et al., *Intravesical oncolytic viral therapy using attenuated, replication- competent, herpes simplex viruses G207 and Nv1020 is effective in the treatment of bladder cancer in an orthotopic syngeneic model.* Faseb J, 2001. **5**: p. 5.
97. Varghese, S., et al., *Preclinical safety evaluation of g207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates.* Hum Gene Ther, 2001. **12**(8): p. 999-1010.
98. Todo, T., et al., *Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus.* Mol Ther, 2000. **2**(6): p. 588-95.
99. Shadan, F.F. and L.P. Villarreal, *Parvovirus-mediated antineoplastic activity exploits genome instability.* Med Hypotheses, 2000. **55**(1): p. 1-4.
100. Chen, Y.Q., et al., *Selective killing of simian virus 40-transformed human fibroblasts by parvovirus H-1.* Cancer Res, 1986. **46**(7): p. 3574-9.
101. Strong, J.E., et al., *The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus.* Embo J, 1998. **17**(12): p. 3351-62.
102. Fields, B.N., *Reoviruses: replication and molecular pathogenesis.* Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi, 1992. **25**(1): p. 56-8.
103. Strong, J.E. and P.W. Lee, *The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection.* J Virol, 1996. **70**(1): p. 612-6.
104. Becker, M.M., et al., *Reovirus sigmaNS protein is required for nucleation of viral assembly complexes and formation of viral inclusions.* J Virol, 2001. **75**(3): p. 1459-75.
105. Bos, J.L., *p21ras: an oncoprotein functioning in growth factor-induced signal transduction.* Eur J Cancer, 1995. **31A**(7-8): p. 1051-4.
106. Bos, J.L., *Ras-like GTPases.* Biochim Biophys Acta, 1997. **1333**(2): p. M19-31.
107. Orntoft, T.F. and H. Wolf, *Molecular alterations in bladder cancer.* Urol Res, 1998. **26**(4): p. 223-33.
108. Luo, Y., et al., *Mutated ras p21 as a target for cancer therapy in mouse transitional cell carcinoma.* J Urol, 1999. **162**(4): p. 1519-26.
109. Connolly, J.L. and T.S. Dermody, *Virion disassembly is required for apoptosis induced by reovirus.* J Virol, 2002. **76**(4): p. 1632-41.
110. Tyler, K.L., et al., *Linkage between reovirus-induced apoptosis and inhibition of cellular DNA synthesis: role of the S1 and M2 genes.* J Virol, 1996. **70**(11): p. 7984-91.
111. Barber, G.N., *Host defense, viruses and apoptosis.* Cell Death Differ, 2001. **8**(2): p. 113-26.

112. Zhang, J.F., et al., *Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy.* Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4513-8.
113. Pavlovich, C.P., et al., *BCG-induced urinary cytokines inhibit microvascular endothelial cell proliferation.* J Urol, 2000. **163**(6): p. 2014-21.
114. Rasouli-Nia, A., et al., *High Raf-1 kinase activity protects human tumor cells against paclitaxel-induced cytotoxicity.* Clin Cancer Res, 1998. **4**(5): p. 1111-6.
115. Xiao, Z., et al., *Characterization of a novel transplantable orthotopic rat bladder transitional cell tumour model.* Br J Cancer, 1999. **81**(4): p. 638-46.
116. Ausubel, F.M., et al., *Current Protocols in Molecular Biology*. 2000, Boston: John Wiley & Sons, Inc.
117. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
118. Schuster, U., et al., *A heterologous in vitro coculture system to study interaction between human bladder cancer cells and fibroblasts.* J Urol, 1994. **151**(6): p. 1707-11.
119. Scheid, M.P. and J.R. Woodgett, *Protein kinases: six degrees of separation?* Curr Biol, 2000. **10**(5): p. R191-4.
120. Coffey, M.C., et al., *Reovirus therapy of tumors with activated Ras pathway.* Science, 1998. **282**(5392): p. 1332-4.
121. Herz, F., A. Schermer, and L.G. Koss, *Short-term culture of epithelial cells from urine of adults.* Proc Soc Exp Biol Med, 1979. **161**(2): p. 153-7.
122. Nawrocki, S., T. Skacel, and I. Skoneczna, *Pharmacotherapy of bladder cancer - practice and prospects.* Expert Opin Pharmacother, 2002. **3**(6): p. 671-9.
123. Sherry, B., *The role of interferon regulatory factors in the cardiac response to viral infection.* Viral Immunol, 2002. **15**(1): p. 17-28.
124. Sturzenbecker, L.J., et al., *Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle.* J Virol, 1987. **61**(8): p. 2351-61.
125. Cardillo, M.R., et al., *Epidermal growth factor receptor, MUC-1 and MUC-2 in bladder cancer.* J Exp Clin Cancer Res, 2000. **19**(2): p. 225-33.